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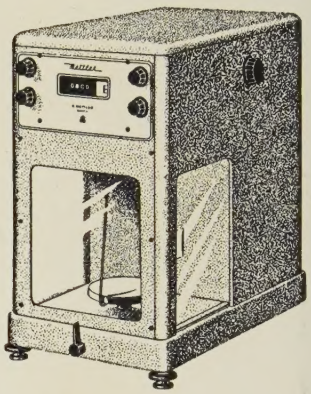

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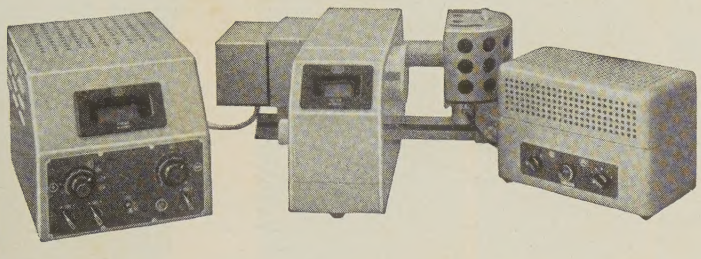

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Immunochemical Analysis of the Surface of the Sea Urchin Egg - an Approach to the Study of Fertilization

By PETER PERLMANN*

Introduction

The sea urchin egg constitutes a classical material for the study of fertilization. As in all metazoa, fertilization of the sea urchin egg 'is the incitement of an egg to development by a spermatozoon, together with the transmission of male hereditary material to the egg'[1]. In its first steps, this process involves (1) the attachment of the fertilizing spermatozoon to the egg, (2) the activating stimulus produced in the egg, and (3) the penetration of the egg by the spermatozoon.

As indicated by a variety of incompatability reactions, the first steps of fertilization are characterized by a high degree of specificity. Ultimately, this specificity depends on one or several highly specific interactions between macromolecules from the sperm and from the egg. Hence, for an understanding of the mechanism of the process it is necessary to know the molecular topography of the surface layers of the gametes and the fine structures, in precise chemical terms, of the molecules which interact.

In this situation specific antibodies, used in combination with common biochemical and biological techniques, could be expected to offer analytical tools of a high power of resolution. As is well known, injection of foreign proteins or polysaccharides into mammals leads to slight structural modifications of certain of the globulins in the serum of the injected animals. Thereby these globulins, which we call antibodies, gain the capacity to react chemically with molecules (i.e. antigens) of the same sort as those which had been injected. Since the reactions between antigens and antibodies are highly specific, antibodies may enable us to determine, *in vitro*, various chemical fine structures in complicated mixtures which at present cannot be distinguished by other means. In addition, a study, in antibody-treated cells, of the interference of the antibodies with special cellular functions, may be useful for an elucidation of the molecular mechanisms operating in these functions[2–7].

Immunological investigations in the field of fertilization are not very numerous and will not be reviewed here. In the sea urchin, the use of antibodies has been introduced by TYLER, aiming to prove the importance of certain substances (fertilizin and antifertilizin) for the process of fertilization[8–12]. A discussion of these results falls beyond the scope of the present article.

Some years ago, the present author started an immunological investigation of the macromolecules which constitute the surface layers of the sea urchin egg, and of the possible significance of these molecules for the process of sperm attachment and activation of the eggs. The study was performed with the gametes of the four Mediterranean sea urchins *Paracentrotus lividus*, *Psammechinus microtuberculatus*, *Arbacia lixula*, and *Sphaerechinus granularis*[13]. The main object of study was *Paracentrotus lividus*. In this article, the course of the analysis will be described and the main results obtained thus far will be discussed.

* The Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden.

[1] LORD ROTHSCHILD, *Fertilization* (Methuen, London 1956).

[2] W. T. J. MORGAN, in A. A. MILES and N. W. PIRIE, *The Nature of the Bacterial Surface* (Blackwell Scientific Publications, Oxford, England 1949), p. 9.

[3] G. W. NACE, Ann. N. Y. Acad. Sci. *60*, 1038 (1955).

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[6] E. A. KABAT, *Blood Group Substances* (Academic Press Inc., Publ., New York 1956).

[7] M. HEIDELBERGER, in Symposium No. 1, IVth Intern. Congress Biochem., Vienna 1958 (Pergamon Press, London 1959).

[8] A. TYLER, Physiol. Rev. *28*, 180 (1948).

[9] A. TYLER, Amer. Naturalist *83*, 195 (1949).

[10] A. TYLER, in B. H. WILLIER, *et al.*, *Analysis of Development* (W. B. Saunders Comp., Publ., Philadelphia and London 1955), p. 170.

[11] A. TYLER and J. W. BROOKBANK, Proc. Natl. Acad. Sci. *42*, 304 (1956).

[12] A. TYLER and J. W. BROOKBANK, Proc. Natl. Acad. Sci. *42*, 308 (1956).

[13] The biological experiments were performed at the Stazione Zoologica of Naples, Italy.

The Morphology of Fertilization

As an introduction to this article, a very brief description of the first steps in fertilization will be given[1,10, 14–17].

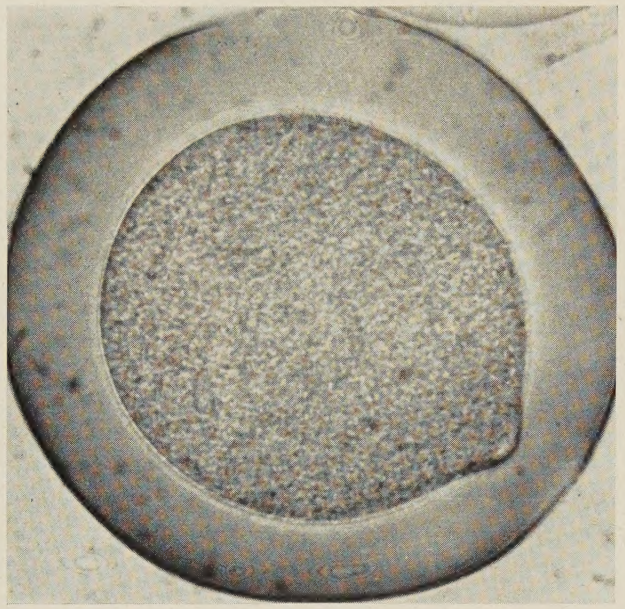

Fig. 1.—Unfertilized sea urchin egg (Echinocardium cordatum). The outer layer is the jelly layer, stained with methylene-blue. The protrusion emerging from the cytoplasm corresponds to the animal pole of the cell. In the eggs of most species, no such protrusions are found. (From IMMERS, unpublished).

Figure 1 shows a mature, unfertilized egg as it occurs free in sea water after shedding. The cell is surrounded by an outer layer, the jelly layer, normally invisible but made visible in the figure by means of staining. The outmost layer of the egg cytoplasm, the cortex, is surrounded by a vitelline membrane and contains the cortical granules; these have been shown to be rich in polysaccharides and possess a species-specific fine structure, apparent in the electron microscope. The layers are shown schematically in Figure 2*a*.

In fertilization, the sperm first passes through the jelly layer. There is evidence that the jelly renders an appreciable proportion of the spermatozoa unfertilizable. Nevertheless, under favorable conditions, some spermatozoa will penetrate the jelly and finally attach to the egg surface. The exact mechanism of this attachment is unknown. However, it seems rather likely that the first contact between sperm and egg is established by means of the tip of the acrosomal filament, a thread-like organella which is expelled from the sperm head at some stage of penetration. Under normal conditions, only one spermatozoon will attach effectively and eventually fertilize the egg. The mechanisms which protect the eggs from polyspermic fertilization are still under discussion.

As soon as the attachment of the sperm is completed a great number of changes are rapidly started in the egg. Thus, from the point of attachment a wave-like change proceeds through the cortex, leading to a continuous expulsion of the cortical granules which are at least partially incorporated in the vitelline membrane. The new membrane, which is lifted up from the egg surface, is called the fertilization membrane. A complete membrane around the egg is normally formed within less than a minute. The space between the membrane and the cortex is called the perivitellin space. Subsequent to the formation of the membrane, a clear, unpigmented layer, the hyaline layer, is formed from the cortex within a few minutes (Figs. 2*b* and *c*; 3).

Simultaneously with these changes of the surface, other marked alterations take place, involving the entire cell. Within 40–50 sec after insemination, the penetration of the fertilizing spermatozoon in the egg can be recorded. At the same time, a total rearrangement

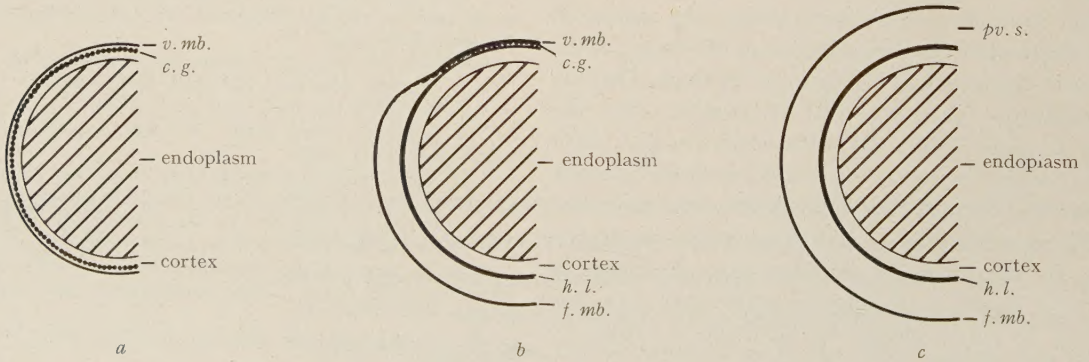

Fig. 2.—Surface layers of the sea urchin egg and their changes upon fertilization. *a*, unfertilized egg; *b*, fertilized egg with partially elevated fertilization membrane; *c*, fertilized egg. – *v. mb.*, vitelline membrane; *c. g.*, cortical granules; *f. mb.*, fertilization membrane; *h. l.*, hyaline layer; *pv. s.*, perivitelline space. – The jelly layer outside the vitelline membrane is not indicated in the Figure. (From RUNNSTRÖM *et al.*[17]).

[14] J. RUNNSTRÖM, Adv. Enzymol. *9*, 241 (1949).

[15] Ch. B. METZ, in A. TYLER *et al.*, *The Beginnings of Embryonic Development* (Publ. Amer. Assoc. Advance. Sci., Washington D. C. 1957), p. 23.

[16] A. L. COLWIN and L. HUNTER COLWIN, in A. TYLER *et al.*, *The Beginnings of Embryonic Development* (Publ. Amer. Assoc. Advance. Sci., Washington D. C. 1957), p. 135.

[17] J. RUNNSTRÖM, B. E. HAGSTRÖM, and P. PERLMANN, in J. BRACHET and A. E. MIRSKY, *The Cell*, Vol. I (Academic Press Inc., Publ., New York 1959).

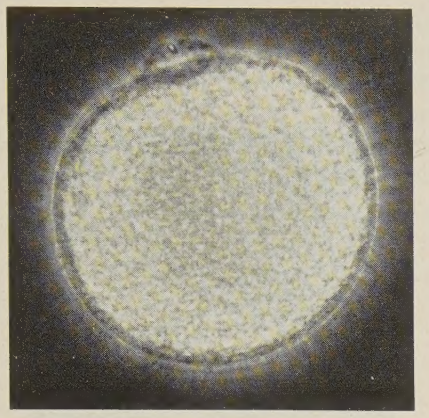

Fig. 3.—Partially fertilized egg of Paracentrotus lividus. Egg and sperm were killed with formalin 10 sec after insemination. By that time only a small membrane blister has been formed. The fertilizing sperm is seen attached to the membrane in that region. (Phase contrast. – From RUNNSTRÖM, unpublished).

of the egg cytoplasm occurs, leading to changes of its optical properties. Subsequent events are aster formation, fusion of male and female pronuclei and, normally after more than one hour, cell division (Figs. 4*a*–*c*).

It must be emphasized that most of the changes described above can also be brought about by various chemical or physical means without the participation of the sperm (*parthenogenetic activation*). Parthenogenesis may lead to membrane and monaster formation and to cell division. However, the ensuing development will frequently be abnormal.

The Immunological Analysis

The Antisera.—The antisera used in the present investigation were obtained from rabbits injected with material from the gametes of various sea urchin species. Antisera, induced by means of injection of *total homogenates from unfertilized eggs*, constituted the most important reagents. Such antisera contain a considerable number of various types of antibodies, directed against the different antigens of the cell. The complexity of the antisera can readily be visualized with the aid of precipitin reactions in agar gel[18,19]. Figure 5*a*, showing the reactions between a saline extract from unfertilized eggs and a number of different anti-egg sera may serve as illustration. The various lines seen in the photograph are each complex and represent a minimum number of specifically different antigen-antibody precipitates[20–23].

The resolving power of the antisera is not restricted to the various macromolecules occurring within the eggs of a particular sea urchin species. Comparison of anti-egg sera obtained with eggs from different species reveal, in addition, a high resolving power of the antibodies with regard to species differences[20,24].

In addition to the anti-egg sera, antisera against *sperm homogenates* or against *isolated egg jellies* were also used. As can be seen from Figure 5*b*, no reactions or at best only very weak reactions took place when extracts from jelly-free eggs were allowed to react with

[18] J. OUDIN, Meth. Med. Res. *5*, 335 (1952).

[19] Ö. OUCHTERLONY, in P. KALLÓS, *Progress in Allergy* (S. Karger, Publ., Basel 1958), p. 1.

[20] P. PERLMANN, Exp. Cell Res. *5*, 394 (1953).

[21] Among these antigens, 7 soluble proteins could be identified further by a combination of biochemical methods and various agar diffusion techniques. Some of these proteins are localized in the yolk granules of the egg whereas others belong to the non-sedimentable part of the cytoplasm. During embryological development these proteins are consumed and synthesized at individual rates. Preliminary accounts of these results have been given elsewhere[22,23].

[22] P. PERLMANN and J. COUFFER KALTENBACH, Exp. Cell Res. *12*, 185 (1957).

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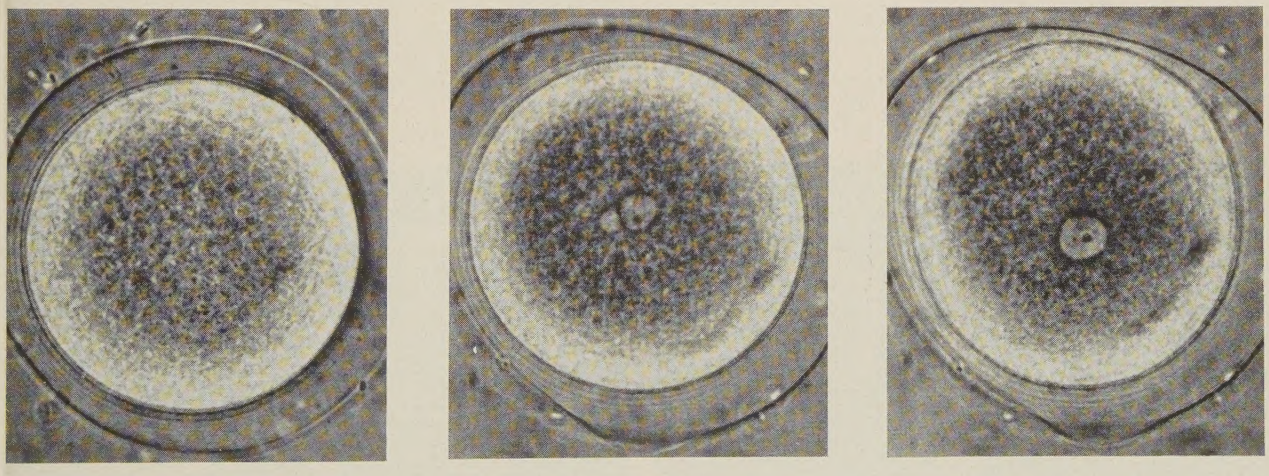
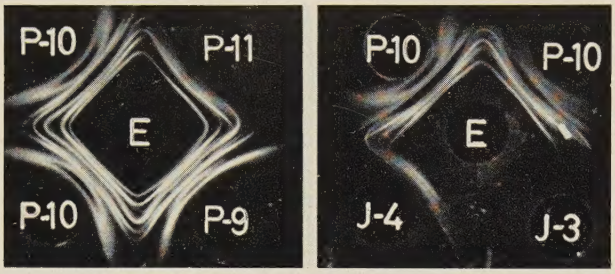


a *b* *c*

Fig. 4.— Fertilization of an egg of Echinocardium cordatum. *a*, Photograph taken 8 min after insemination. Jelly layer is invisible. The protrusion of the fertilization membrane corresponds to the animal pole, seen in Figure 1. Note supernumerary sperm attached to the fertilization membrane; *b*, The same egg, 22 min after insemination. Male and female pronuclei now visible close together near the centre of the cell; *c*, The same egg, 40 min after insemination. The pronuclei have now fused. Approximately 10 min later the zygotic nucleus disapears while dividing. (Phase contrast. – From IMMERS, unpublished).

two such anti-jelly sera. Experiments of this kind demonstrated an appreciable difference in antigenic constitution of egg cytoplasm and jelly layer. Similarly, differences are found when the antigens in extracts from the eggs are compared with those in extracts from the sperm [20].



a *b*

Fig. 5.—Immunological precipitations in agar. Dark field photographs, taken after 2 days incubation at 38°C, followed by 2 weeks in the refrigerator. *E*, soluble part of egg homogenate (*Paracentrotus lividus*), diffusing from reservoirs in the centre; P-9, P-10, P-11, antisera, prepared in different ways, against unfertilized, jelly-free eggs; J-3, anti-jelly serum, obtained with a purified jelly preparation; J-4, anti-jelly serum obtained with a crude preparation, contaminated with egg antigens. Each antiserum is the serum pool from 5 rabbits. The various antisera diffused from the peripheral reservoirs. For further explanations see text. (From PERLMANN [27]).

The Response of the Eggs to Antiserum.—For the study of the antigens concerned with fertilization, living eggs were exposed to antiserum. Antigen-antibody reactions taking place in the eggs then led to morphological or physiological alterations which could be recorded. The procedure is shown schematically in Figure 6.

Exposing a small number of eggs, for example 500, to a standard amount of antiserum, e.g. 0·5 ml, renders them completely unfertilizable; such eggs will not react with the sperm even after removal of the antiserum. Moreover, they will be microscopically altered.

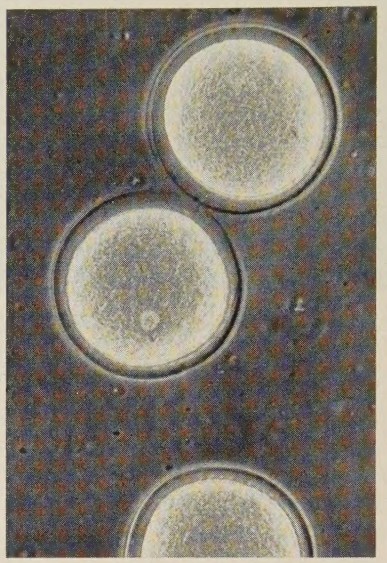


Fig. 7.—Jelly precipitation. Unfertilized eggs of *Paracentrotus lividus*, treated with homologous anti-jelly serum. Jelly layers precipitated and contracted. (Phase contrast).

Thus, frequently a precipitation of the jelly layers is obtained; the affected jellies have changed optical properties and become visible in the microscope. In addition, the precipitated layers very often contract. Jelly precipitated eggs always agglutinate (Figs. 7, 8) [25–27].

[25] P. PERLMANN, Exp. Cell Res. *6*, 485 (1954).
[26] P. PERLMANN, Exp. Cell Res. *10*, 324 (1956);
[27] P. PERLMANN, Exp. Cell Res. *13*, 365 (1957).

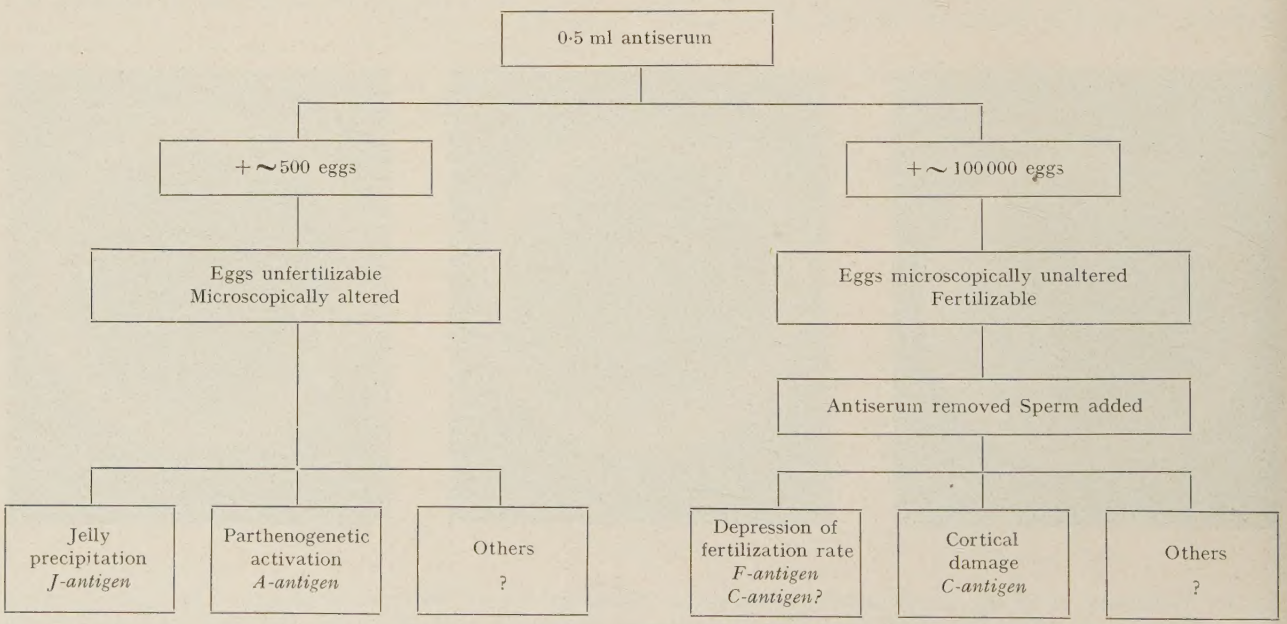


Fig. 6.— Procedure applied for analysis of various surface antigens. Aliquots of, e.g., 0·5 ml antiserum were either mixed with 500 eggs, or with 100,000 eggs. The various response reactions indicated in the Figure served for recording the different activities of the antisera. For further explanations see text.

Exposure of the eggs under the same conditions often leads to another response reaction which may be considered as being the most important in the present connection. In the eggs from a considerable proportion of the females, anti-egg serum induces parthenogenetic activation. Such activated eggs develop cortical reactions, leading to the formation of irregular membranes or membrane blisters, nuclear activation and monaster formation. Frequently, one or more irregular cell divisions take place also (Figs. 9, 10) [25–27].

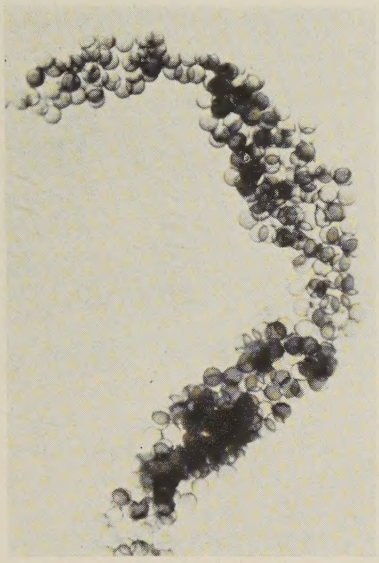


Fig. 8.—Agglutination of jelly precipitated eggs. Eggs from the same experiment as in Figure 7, photograph taken 1 h later. The layers now contracted to very narrow rims, not visible at the magnification used for the photograph. Eggs strongly agglutinated.

The extent of jelly precipitation and activation is strictly dependent on the ratio in which eggs and antiserum are mixed. When these ratios are too high, as shown in the right part of Figure 6, the eggs appear completely normal as judged from microscopic observations. Nevertheless, alterations are found when their behavior in fertilization is studied after removal of the antiserum.

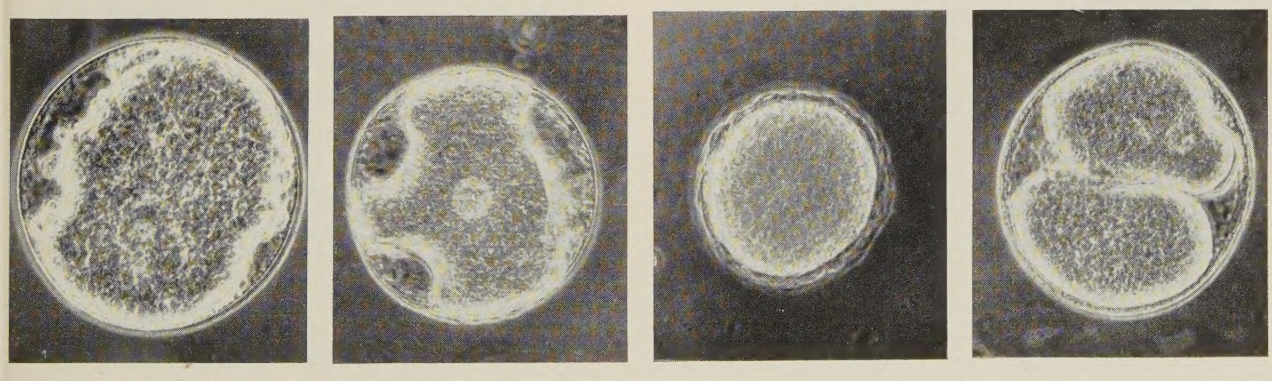
Most characteristical for eggs, fertilized after pretreatment with antiserum, are certain typical damages to the cortex. The fertilization membrane often remains attached to the egg surface and frequently only membrane blisters are formed. The membrane appears precipitated and the dissolution of the cortical granules is more or less inhibited (Figs. 11, 12). This also leads to a high tendency in the eggs for polyspermic fertilization and to severe disturbances of development [27,28].

Treatment of the eggs prior to fertilization with anti-egg serum reduces their fertilizability. One expression of this action is a depression of the 'rate of fertilization'. This rate can be measured by determining the proportion of eggs fertilized after various periods of egg-sperm interaction where fertilization has been interrupted by killing the sperm but not the eggs (Fig. 13) [29,30,27,31].

The Analysis of the Response Reactions.—Extensive series of experiments showed that sera from normal rabbits were inactive. Hence, it could be inferred that the effects described above were due to the action on the eggs of specific antibodies. In addition, comparison of the action of antiserum on the eggs from different sea urchin species revealed a surprising species specificity of the antibodies, as exemplified in Figure 13. Anti-sperm sera could be shown to be inactive also. For details see [26,27,31].

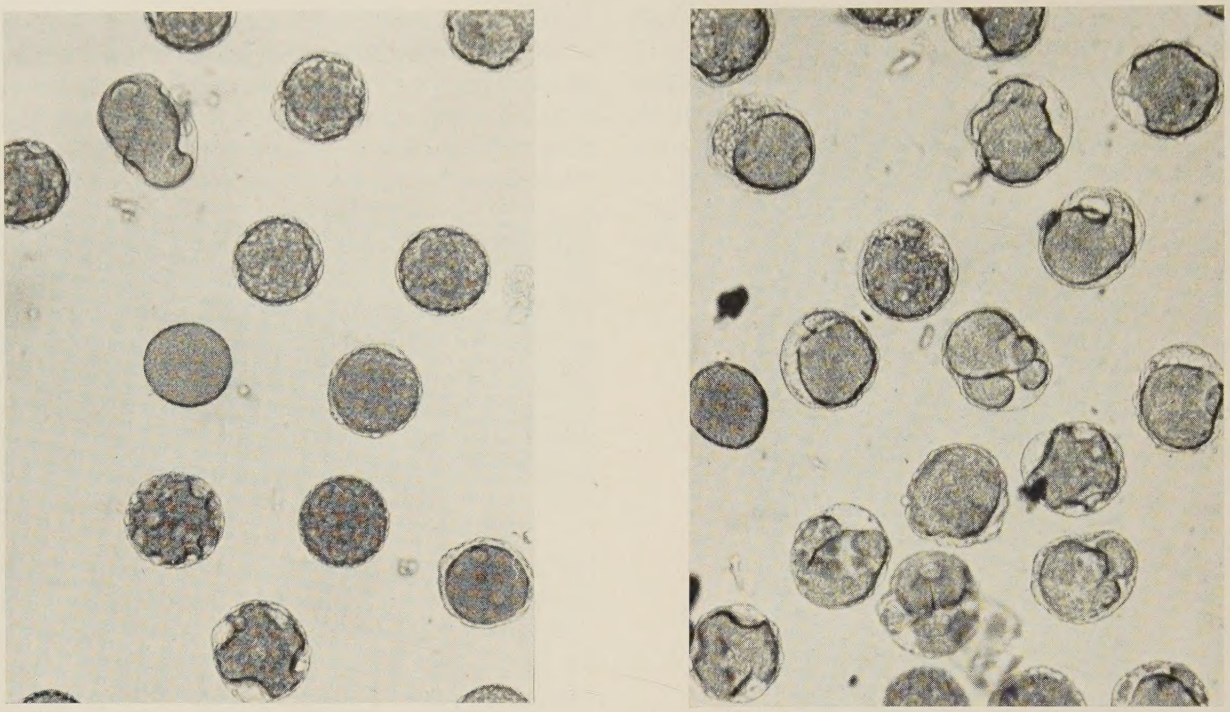
The next aim of the analysis was to achieve a resolution of the complex effects of antiserum into single components. As a first step, the nature of different variables governing the various responses of the eggs was studied. Subsequently, the dose-response relationships, valid under specified experimental conditions, were established for each of the responses described above. The findings from such experiments

[28] P. Perlmann and B.E. Hagström, Exp. Cell Res. *12*, 418 (1957).
[29] Lord Rothschild and M. M. Swann, J. exp. Biol. *28*, 403 (1951).
[30] B. E. Hagström and B. Hagström, Exp. Cell Res. *6*, 479 (1954).
[31] P. Perlmann and B. E. Hagström, Exp. Cell Res. Suppl. *3*, 274 (1955).



a *b* *c* *d*

Fig. 9.— Parthenogenetic activation. Unfertilized eggs of *Paracentrotus lividus*, treated with homologous anti-egg serum. *a*, Egg with incompletely elevated, irregular membrane; *b*, Egg with pronucleus swollen, migrated to the centre of the cell; *c*, Monaster egg, with small irregular membrane blisters; *d*, Slightly abnormal 2-cell stage (Phase contrast).



a *b*

Fig. 10. — Parthenogenetic activation. Unfertilized eggs of *Paracentrotus lividus*, parthenogenetically activated with homologous anti-egg serum. *a*, Photograph taken 2 h after beginning of antiserum treatment; *b*, Photograph from the same experiment, taken 12 h after beginning of serum treatment. Many eggs show beginning cytolysis.

constituted the basis for a comparative study where the activities of a number of differently prepared antisera were tested. The results have been summarized in Figure 14. They indicated, although they did not prove, that the responses were due to the reactions of at least 3–4 separate antibodies, with their corresponding antigens [27].

The validity of these assumptions was finally tested with the aid of *antiserum inhibition techniques* [32,6]. In these, use was made of specific absorption of antibodies prior to testing the antisera for their activities. Thus, when extracts or fractions, obtained from the eggs, are added to the antisera, antibodies are precipitated or

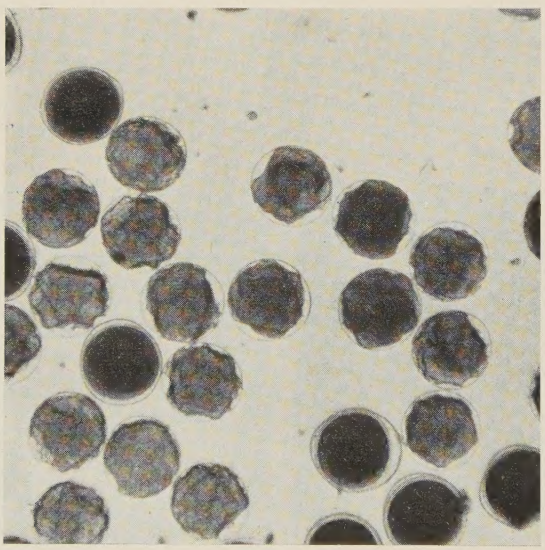


Fig. 11.—Cortical damage. – Fertilized eggs of *Paracentrotus lividus*, pretreated with homologous anti-egg serum. Egg and sperm killed by addition of formalin approximately 10 min after insemination. For explanations see text.

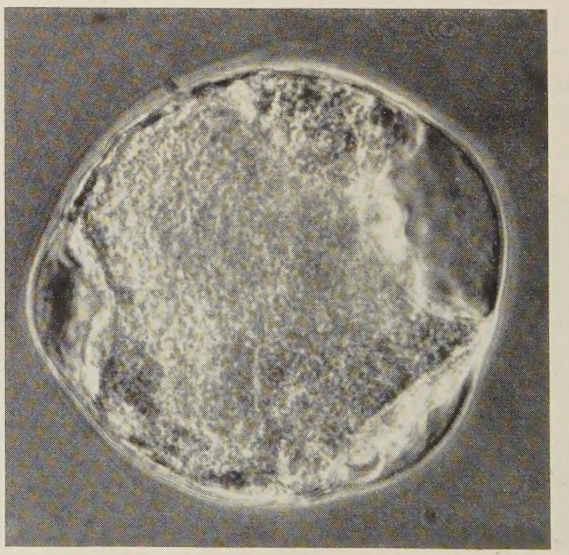


Fig. 12.—Cortical damage. Egg from the same experiment as in Figure 11 (Phase contrast).

[32] K. LANDSTEINER, *The Specificity of Serological Reactions* (Harvard University Press, Cambridge, Mass. 1946).

bound by antigen. When tested on the eggs, various activities of such antisera may be inhibited, depending on which antibodies were eliminated by absorptions. The type of results obtained is exemplified in Figure 15.

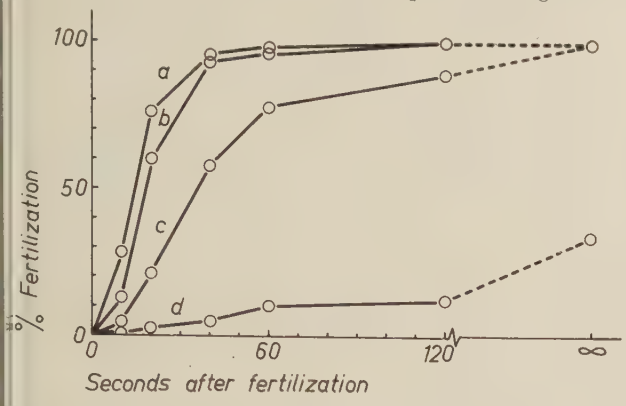


Fig. 13.—Depression of fertilization rate. Eggs of *Paracentrotus lividus*, fertilized after pretreatment with various sera. *Abscissa*, time in seconds of egg-sperm interaction. *Ordinate*, % fertilized eggs. Each symbol represents 200 counted eggs. The various curves correspond to different pretreatments of eggs from the same batch *a*) eggs pretreated with antiserum against eggs from *Arbacia lixula*; *b*) eggs pretreated with serum from normal rabbits; *c*) eggs not pretreated; *d*) eggs pretreated with antiserum against eggs from *Paracentrotus lividus* (From PERLMANN and HAGSTRÖM[31]).

Such inhibition experiments proved in fact that there were four different antigens involved in the response reactions mentioned in this section. In addition, certain conclusions could be drawn concerning the chemical nature of the antigens[33,34].

The antigens which will be discussed in the following are those involved in the response reactions shown in Figure 6. Other responses, e.g. osmotic alterations in the surface of the unfertilized eggs[26], induction of

[33] P. PERLMANN and H. PERLMANN, Exp. Cell Res. *13*, 454 (1957).
[34] P. PERLMANN and H. PERLMANN, Exp. Cell Res. *13*, 475 (1957).

Anti-serum	Activity			
	Jelly precipi-tation	Activation	Fertilization rate depression	Cortical damage
P–9	+	+ + ±	+ + +	+ + +
P–10	±	+ +	+ +	+ + ±
P–11	±	+ + +	+ + ±	+
J–3	+ + +	±	0 ?	0
J–4	+ + +	+ + ±	+	±
Sp–6	0	0	0	0

Fig. 14.—Comparison of various activities of a number of anti-Paracentrotus sera. P-9, P-10, P-11, antisera against jelly-free eggs. The mode of preparation of antiserum P-11 was different from that used for preparation of P-9 and P-10; J-3, anti-jelly serum obtained with a purified jelly preparation; J-4, anti-jelly serum obtained with a crude jelly preparation (the precipitin-reactions obtained with these antisera are shown in Figure 5); Sp-6, anti-sperm serum. Each antiserum the serum pool from 5 rabbits. 0 = inactive, ± to + + + = very weak to strongly active (From PERLMANN[27]).

polyspermy and inhibition of cytoplasmic cleavage[28], have not yet been analyzed and will not be treated in this article. The details given below concern the eggs of *Paracentrotus lividus* only. Although eggs from other species respond similarly when exposed to homologous antiserum, certain characteristic differences do exist[26,31]. The antigens of the other species have not yet been analyzed in detail.

Jelly Precipitation; the J-Antigen

With the eggs of *Paracentrotus lividus*, the comparison of the jelly-precipitating potencies of different antisera suggested that the effect was due to the reaction between antibodies and a particular jelly antigen (Fig. 14)[27]. This could be fully confirmed by means of inhibition experiments; thus, absorption of the antisera

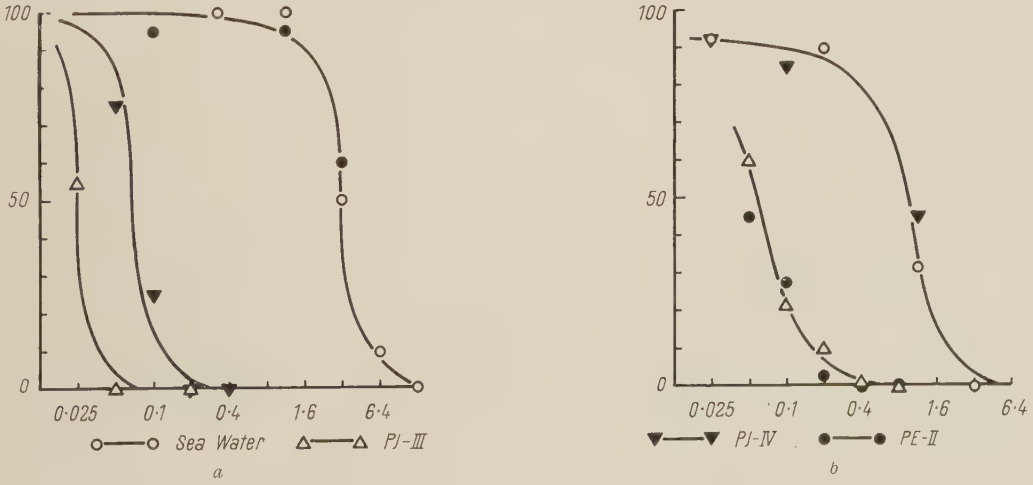


Fig. 15.—Inhibition of jelly precipitation or of activation of Paracentrotus eggs after absorption of antiserum. *Abscissae*, ml of sea water or of various antigen solutions used for absorption of one-ml aliquots of an anti-jelly serum (*a*), or an anti-egg serum (*b*). For testing, 0·8-ml aliquots of the absorbed (or diluted) antisera were mixed with aliquots of 0·2 ml sea water, each containing 500 reactive eggs. *Ordinates*, (*a*) % jelly precipitated or (*b*) % activated eggs. Each symbol represents 200 counted eggs. Antigen solutions used for absorption: *PJ-III*, *PJ-IV*, solutions of 2 jelly preparations, corresponding to ~23,000 or ~7000 eggs/ml, respectively; *PE-II*, soluble part of egg homogenate, made from jelly-free eggs, 32,000 eggs/ml. For further details[33].
(From P. PERLMANN and H. PERLMANN[33]).

with various isolated jelly preparations or with homogenates of jelly-free eggs revealed a high inhibitory capacity of the former preparations whereas the latter were inactive (Fig. 15*a*) [33].

The antigen, which thus must be part of the jelly layer, is called the *J-antigen*. Inhibition experiments showed that it is heat-stable (100°C) between pH 4–8. At pH3·5, it is susceptible to treatment with periodate as well as to heating to 100°C. Treatment with HCl of pH 1 at room temperature reduces the J-activity. The specificity is completely destroyed by 4 h heating to 100°C at pH 1 [33]. Inhibition experiments with a number of single sugars suggest that L-fucose is an essential part of the antigenic determinants (unpublished experiments).

The jelly layer of the sea urchin egg consists of sulfated polysaccharides in combination with protein or peptides [14]. The jelly layer of *Paracentrotus lividus* contains L-fucose as the main sugar and in addition glucose in small and variable quantities [35,36]. The data given above indicate that the J-antigen is an integral part of the main polysaccharide of the jelly layer.

The nature of the jelly precipitation is not understood. The coagulation no doubt represents a common way for the jelly to respond to many kinds of unrelated chemical agents, e.g. basic proteins or substances which can be extracted from the gametes themselves [10,14]. It remains to be established which chemical groupings of the jelly layer are reacting in the various cases.

The response of the eggs is very variable and entirely unresponsive eggs are always found. The reactivity of the eggs seems to be related to their state of maturity and varies during the spawning season [26,27]. Since this variability seems to be correlated to the capacity of the jellies to fix antibodies (unpublished experiments), it might indicate that chemical alterations are taking place in the layers during maturation.

The jelly layer of the sea urchin egg is the bearer of a sperm-agglutinating principle, fertilizin [37], which is species-specific in its action. The agglutination is assumed to be brought about by means of an 'immunological' reaction between the fertilizin of the egg and the corresponding antifertilizin in the sperm [1,8,9,15]. From the data at hand it is not apparent whether or not the J-determinants of the jelly substance are identical with the chemical structures exhibiting fertilizin activity. So far, attempts to inhibit fertilizin-induced sperm agglutination with J-antibodies have been without success [38]. Pretreatment of the eggs with J-antibodies is seemingly without effect on fertilization, even when the antibodies are applied over a wide range of concentrations [27], also [34].

[35] E. VASSEUR, Acta chem. scand. *2*, 900 (1948).
[36] E. VASSEUR and J. IMMERS, Ark. Kemi *1*, 39 (1950).
[37] F. R. LILLIE, *Problems of Fertilization* (University of Chicago Press, Chicago 1919).
[38] U. NYBERG and P. PERLMANN (to be published).

Parthenogenetic Activation; the A-Antigen

In *Paracentrotus lividus* both anti-egg and anti-jelly sera may induce activation [26,27]. However, as shown in Figure 14, the jelly-precipitating and the activating potencies of the antisera were unrelated. The parthenogenetic antibodies could thus be assumed to differ from the jelly-precipitating antibodies. Consistent results were obtained in inhibition experiments; these conclusively demonstrated that the activating antibodies were directed against one or several antigens derived from the cytoplasm of the eggs; however, considerable amounts of these antigens may sometimes occur in isolated jelly substances (Fig. 15*b*) [33].

Further inhibition experiments indicated that the total inhibitory activity of the egg homogenates could be recovered in their soluble fraction. The antigen did not belong to the soluble proteins which are destroyed by the action of proteolytic enzymes. Furthermore, it withstands heating to 100°C both at pH 8 and at pH 3·5. On the other hand, it is partially destroyed by the action of periodate and by heating to 100°C at pH 1 (Fig. 16) [33].

When the soluble part of a boiled homogenate is studied in the electrophoresis apparatus, 3 main components of distinct mobilities are found [33]. These can be separated and tested in inhibition experiments. All A-activity is recovered from the slowest moving fraction, from which it can be further purified by means of precipitation with ethanol. Another way of partial purification is to precipitate the crude extract with HCl at pH 2. The precipitate then contains all the nucleic acid of the extract but only a small fraction of the antigenic activity. About 90% of the latter can instead be recovered from the supernatant from which it can again be purified further by means of ethanol. The methods are summarized in Table I; some qualitative chemical data of A-active preparations, obtained in these ways, are given in Table II.

Table I. – Preparation of A-antigen (*Paracentrotus lividus*)
Unfertilized eggs, freed from jelly layer at pH 5·2, homogenized, treated at 100°C for 4 h (pH 7·8), centrifuged. A-activity in supernatant.

Procedure 1

A-active fraction separated electrophoretically, precipitated with ethanol.

Procedure 2

HCl added → pH 2, centrifuged

Residue	*Supernatant*
10% of A-activity	90% of A-activity, precipitated with ethanol

Table II.—Chemical properties of A-antigen

Easily soluble in water, insoluble in ethanol. Not susceptible to the action of trypsin or pepsin. Heat stable at pH 3·5–8. Slowly hydrolyzed at pH 1–2 at 100°C. Partially susceptible to IO_4^- (pH 3·5,50°C). Electrophoretic mobility $-1{\cdot}6 \times 10^{-5}$ cm^2 × volt^{-1} × sec^{-1} (pH 7·1, phosphate, $\mu = 0{\cdot}1$).

Some qualitative data on partially purified A-antigen

Glucose +	Hexosamine –	Ribonucleic acid –
Mannose +	Sialic acids –	Sulfate +
Galactose –	Amino acids +	Phosphate –
Fucose –		

The results led to the conclusion that activation, in all antisera, was caused by a particular antigen-antibody reaction. The antigen involved has been called the *A-antigen*. The A-antigenic determinants are of a carbohydrate nature but belong to a molecule different from the J-antigenic molecule. This statement involves no implications as to the nature of the molecule *in toto*. Thus, the polysaccharide may well be combined with peptides and/or with lipids; in addition, in the living cell it might be closely associated with other polysaccharides. The data given in Table II do not yet allow any conclusions as to the composition of the antigen. However, it may be noted that glucose seems to be the only carbohydrate common to both the antigenically active fraction of the egg extract and the isolated jelly layers which are A-active also. This might suggest that glucose, perhaps in combination with sulfate, is an integral constituent of the A-antigenic structures.

The distribution of the A-antigen between the egg cytoplasm and the jelly layer clearly suggests its localization in the cytoplasmic surface of the cell. The existence of substances of this type in the egg surface is well established[39–41]. The antigen seems to be bound in a labile fashion and hence may diffuse readily into the jelly layer[42]. A diffusion, occurring in the living egg, might at least partially explain the considerable variability in the parthenogenetic response towards antiserum of eggs of different origin or state of maturity, and of normal eggs or jelly-less eggs[26,27].

It is of special interest that the eggs can be activated by means of a particular antigen-antibody reaction, different from a variety of other such reactions which take place in or near the surface of the antiserum-treated cells. Although it contrasts with the commonly found inhibitory or cytotoxic effects of antibodies on cells[3], this response is consistent with the functional organization of the egg surface. The rearrangement of acid polysaccharides in the egg surface in connection with egg activation[41], and the activation of proteolytic enzymes[43] may offer analogies to certain events that are known to follow upon the immunological trigger reaction in anaphylaxis and allergy[44–48]. Thus, the stimulating action of the antibodies may be mediated

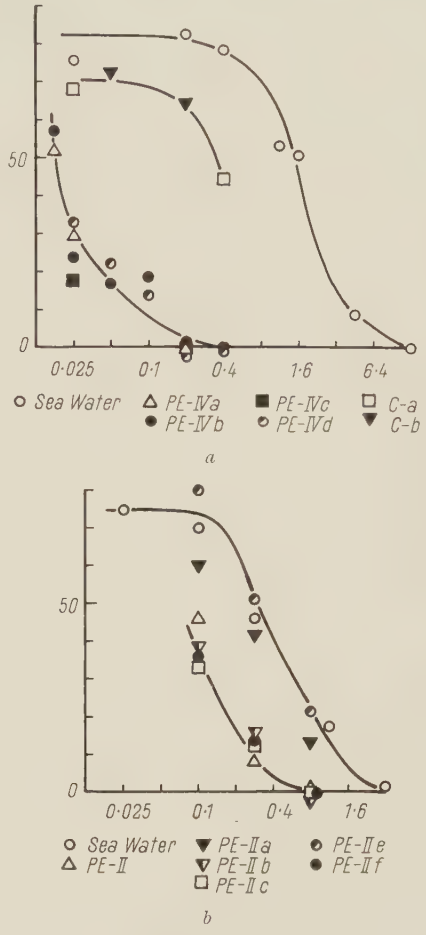


Fig. 16.—Two typical inhibition experiments bearing on the chemical nature of the A-antigen of the Paracentrotus egg. *Abscissae*, ml of sea water or various antigen solutions used for absorption of one-ml aliquots of an anti-egg serum. For testing, 0·8-ml aliquots of the absorbed (or diluted) antisera were mixed with aliquots of 0·2 ml sea water, each containing 500 reactive eggs. *Ordinates*, % activated eggs. Each symbol represents 200 counted eggs. Antigen solutions used for absorptions: (*a*) *PE-IV*, homogenate made from jelly-free eggs; *PE-IVa*, treated with trypsin; *PE-IVb*, corresponding control without trypsin; *PE-IVc*, treated with pepsin; *PE-IVd*, corresponding control without pepsin; *C-a*, *C-b*, controls containing enzymes and enzyme inhibitors but no egg homogenates. (*b*) *PE-II*, homogenate made from jelly-free eggs; *PE-IIa*, treated with periodate, pH 3·5, 50°C, 240 min; *PE-IIb*, heated to 50°C, pH 3·5, 240 min; *PE-IIc*, heated to 100°C, pH 3·5, 240 min; *PE-IIe*, heated to 100°C, pH 1, 240 min; *PE-IIf*, kept at 20°C, pH 1, 240 min. For further details[33]. (From P. Perlmann and H. Perlmann[33].)

through the immunological inactivation of an enzyme inhibitor, viz. the A-antigen, and the subsequent activation of a number of proteolytic enzymes. This

[39] L. Monné and S. Hårde, Arkiv Zool. (2) *1*, 487 (1951).
[40] J. Immers, Arkiv Zool. (2) *9*, 367 (1956).
[41] J. Runnström and J. Immers, Exp. Cell Res. *10*, 354 (1956).
[42] I. Motomura, Sci. Rep. Tohoku Univ., 4th Ser. *18*, 554 (1950).
[43] G. Lundblad, Arkiv Kemi *7B*, 127 (1954).
[44] P. G. H. Gell, Biochem. Soc. Symposia (Cambridge, Engl.) No. 10, 63 (1953).
[45] M. Rocha e Silva, *Histamine, its Role in Anaphylaxis and Allergy* (Charles C. Thomas Publisher, Springfield, Ill. 1955).
[46] G. Ungar, E. Damgaard, and F. P. Hummel, J. exp. Med. *98*, 291 (1953).
[47] G. Ungar, Intern. Arch. Allergy appl. Immunol. *4*, 258 (1953).
[48] G. Ungar and E. Damgaard, J. exp. Med. *101*, 1 (1955).

explanation would be in line with the hypothesis of egg activation developed by RUNNSTRÖM[49, 41, 17].

In the eggs, activated by means of antiserum, the spreading of a submicroscopic 'fertilization wave', believed to induce the *microscopic* cortical reactions[50–54, 1, 17] seems to be inhibited. Most likely, this inhibition is, at least partially, due to the action of unrelated antibodies (cf. below) which affect other antigens in the cortical ground substance or in the cortical granules. Owing to this elimination of the self-propagating mechanism, the stimulating antigen-antibody reactions must probably cover a wide region of the egg surface in order to induce a visible response.

There is little doubt that the A-antigen is an important constituent among the molecules involved in the normal activation of the egg. We may probably assign to it a key role as initiator of activation in fertilization. If so, this assumption does not necessarily imply *that the possible reaction between the A-antigen and the sperm involves the A-active structures, nor that it at all involves an 'immunological' reaction.* The reaction might well be of enzymatic nature.

Cortical Damage; the C-Antigen

As mentioned above, certain characteristic anomalies in the cortical reactions of fertilized eggs are among the most striking features after pretreatment with antiserum (Figs. 11, 12)[27, 28]. No obvious correlation could be detected between the jelly-precipitating or the activating potencies of the antisera on one hand and their cortex-damaging potencies on the other. It was of interest to note that the antisera with weak or non-existent cortex damaging potencies (P-11, J-3, J-4) were devoid also of antibodies reacting with certain soluble proteins in agar-diffusion experiments (Figs. 5 and 14)[27].

Inhibition experiments indicated that the antigen involved in this response is a soluble egg antigen, not detectable in the jellies. The antigenic activity is not destroyed with trypsin. On the other hand, the antigen does not withstand heating at pH 7–8 and is thus chemically entirely different from the J- and A-antigens described above. This antigen, which may be a protein, is called the *C-antigen*[34].

Pretreated and fertilized eggs with cortical damage closely resemble eggs which have been activated by certain of the antisera. It seems reasonable to assume that the pronounced damage of the breakdown of the cortical granules and of membrane formation in both cases may be induced by the antibodies reacting with the C-antigen. In any event, judging from the type of response, a superficial localization of the C-antigen may be postulated. It may possibly be contained in the cortical granules. In any case, it certainly is of importance in determining the course of the cortical reaction. Moreover, it is likely that the increased tendency for polyspermic fertilization induced by certain antisera[28], is due to the reactions of antibodies with the C-antigen.

Fertilization Rate Depression; the F-Antigen

Comparison of the potencies for depressing fertilization rate of various antisera also showed that neither the antibodies against the J-antigen nor those against the A-antigen exert much activity. Moreover, the cortex-damaging and the fertilization-rate-depressing potencies of the anti-egg sera appeared to be unrelated (Fig. 14)[27].

In inhibition experiments, isolated jelly preparations were of low inhibitory capacities whereas equivalent amounts of egg homogenate were strong inhibitors. Hence, the antibodies responsible for fertilization rate depression are also directed against antigens derived from the cytoplasm of the eggs. Absorption with sperm homogenate had no effect (Fig. 17*a*)[34].

The rate-depressing antigens seem to be only partially soluble in water or saline. They are not attacked by trypsin. Heating (100°C, 4 h) reduces the inhibitory capacity of the homogenates without destroying it completely (Fig. 17*b*). The data were interpreted to mean that one ore more additional heat-stable antigens, different from the J- and A-antigens, are probably involved in fertilization rate depression. The antigen(s) has (have) been called the *F-antigen.* The heat labile factor involved in fertilization rate depression may be identical with the C-antigen[34].

The depression of the rate of fertilization suggests a localization of the F-antigen in the cytoplasmic surface also. Since it is heat stable it might well belong to the structures of the cortex assumed to be rich in polysaccharides or mucoproteins[40, 41, 55].

The depression of the rate of fertilization, following the immunological reactions, suggests that the F-antigen might constitute a specific sperm receptor of the egg surface. However, fertilization rate depression is not a simple response and several unrelated mechanisms are likely to be at work[29, 1, 27]. For example, the fertilization rate depression, brought about by the reactions of antibodies with *heat labile* antigens (the C-antigen?), may be due to the inhibition of a cortical mechanism determining the physical or chemical reactivity of the surface. Therefore, in the rate experiments, an attached spermatozoon may sometimes be killed before it succeeds in starting a visible reaction in

[49] J. RUNNSTRÖM, Exp. Cell Res. Suppl. *1*, 469 (1949).
[50] J. RUNNSTRÖM and G. KRISZAT, Exp. Cell Res. *3*, 419 (1952).
[51] M. SUGIYAMA, Biol. Bull. *104*, 210 (1953).
[52] M. SUGIYAMA, Biol. Bull. *104*, 216 (1953).
[53] M. SUGIYAMA, Exp. Cell Res. *10*, 364 (1956).
[54] R. D. ALLEN, Exp. Cell Res. *6*, 412 (1954).
[55] E. NAKANO, Embryologia *3*, 139 (1956).

the egg. This mechanism may be quite independent from a possible sperm receptor mechanism, instru-

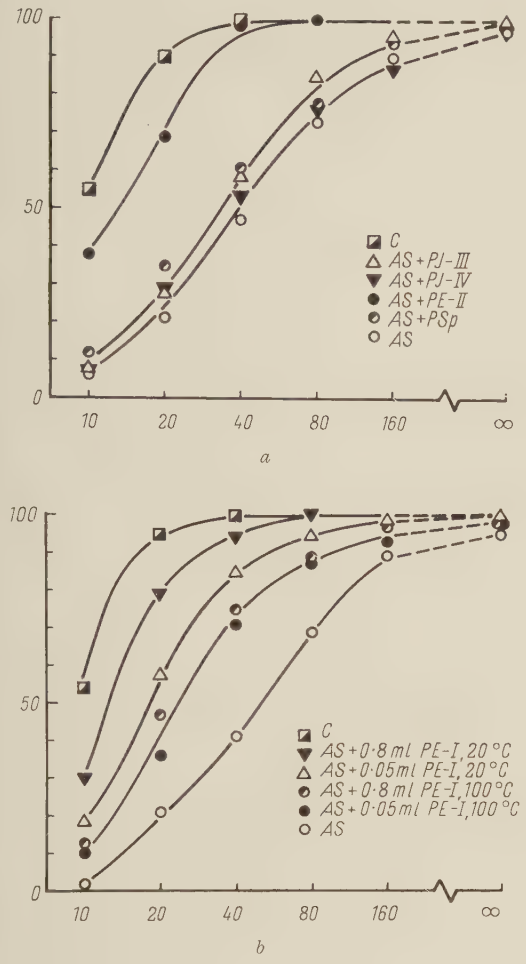


Fig. 17.—Fertilization rate of *Paracentrotus* eggs, pretreated with various absorbed or unabsorbed samples of an anti-egg serum. *Abscissae*, time in seconds of egg – sperm interaction; *Ordinates*, % fertilized eggs. Each symbol represents 200 counted eggs. The various curves correspond to different pretreatments of the eggs as indicated in the Figures. *C* = eggs pretreated with normal rabbit serum; *AS* = antiserum. (*a*) *PJ-III*, *PJ-IV*, jelly preparations, *PE-II*, homogenate from jelly-free eggs, used for absorption of the antiserum. Amounts corresponding to equivalent numbers of eggs were taken for absorption in all cases. *PSp*, sperm homogenate. (The jelly preparation *PJ-III*, which was of a very low inhibitory capacity in this experiment was a strong inhibitor of activation due to a high content of A-antigen.) (*b*) *PE-I*, *20°C*, homogenate from jelly-free eggs, untreated; *PE-I*, *100°C*, aliquot of the same homogenate, heated to 100°C for 240 min at pH 8; 0·05 ml, 0·8 ml, volumes of antigen solutions added to one-ml aliquots of antiserum for absorption. The various curves in this diagram are significantly different from each other. For the statistical treatment of the data of fertilization rate experiments[27]. For further details[34].
(From P. Perlmann and H. Perlmann[34].)

mental during the attachment of the sperm and involving other, *heat-stable* factors, such as the F-antigen.

The Specificity of Sperm Attachment

In order to explain the specificity of fertilization, Lillie introduced the immunological concept by assuming sperm attachment to be mediated by means of antigen-antibody like reactions between complementary molecules. The basis for his assumption was the finding of the sperm agglutinating fertilizin, derived from the eggs[37]. As already mentioned above, fertilizin has later been identified as a component of the jelly layers of the eggs[10,14,15].

Fertilizin has long been believed to have an *improving* effect on the process of sperm attachment and to be indispensable for fertilization[56,8,10]. However, except for certain data indicating an improving action of jelly substance on various activities, e.g. motility or oxygen consumption, of the sperm[57,58,1], there is at present no conclusive evidence in favour of any improving action in fertilization, neither of the jelly layer nor of its fertilizin. On the contrary, there has accumulated an increasing body of evidence indicating general inhibitory effects of dissolved jelly substance as well as of the jelly layer *in situ* on various phases of fertilization or egg activation[59–65]; also [17].

Whatever the biological significance of the 'immunological' fertilizin-antifertilizin reaction, it seems quite evident that it cannot be as predominant in determining the specificity of fertilization as sometimes claimed. Obviously, in the present connection, the immunological concept cannot be taken too literally—a fact which for instance was well recognized by Lillie[37]. Whereas the reactions between antigens and antibodies are confined to rather restricted areas on the surface of molecules, the specificity of the process of sperm attachment must be assumed to be more complex. It may involve, in its first stages, electrostatic attraction between charged groups, arranged in self-repeating patterns on the surface of the gametes. Bivalent cations may play a rôle in binding together identically charged areas on the surfaces of the gametes[66,67]. Upon closer contact between the molecules, irreversible attachment of a higher degree of specificity may follow, involving various specific patterns of short range forces and steric factors. The final step of egg activation and penetration may require a spatially oriented union between an enzyme, for example in the acrosomal filament of the sperm[16,68,69], and a prosthetic group at the receptor sites of the eggs. Analogous conditions may be found in the fields of bacteriophage attachment and of virus

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[69] B. Afzelius and A. Murray, Exp. Cell Res. *12*, 325 (1957).

hemagglutination [66,70–81]. Whatever the exact mechanism, it appears reasonable to consider the existence of particular requirements of 'complimentariness', involving different molecules, during the various stages of the process. It remains to be established, whether or not the fertilizin-antifertilizin reaction is involved, and, if so, what its rôle and its position in the sequence may be.

The present investigation has given evidence for the occurrence of four structurally different types of macromolecules in the surface layers of the sea urchin egg. At least three of these seem to have particular and important functions in connection with sperm attachment and egg activation. In addition, there are indications of the presence of additional factors, not yet determined but probably available for analysis. Finally, functionally similar but structurally different molecules are present in the eggs of other sea urchins. It may be hoped that further immunochemical studies of the various macromolecules participating in fertilization will permit a direct approach to the problems discussed in this section.

Zusammenfassung

Im vorliegenden Aufsatz wird über die Ergebnisse immunologischer und immunochemischer Untersuchungen der Oberflächenstrukturen der Eier einiger mediterraner Seeigelarten berichtet. Die Eier werden Antiserum ausgesetzt, das durch Einspritzungen von Seeigel-Gametenmaterial in Kaninchen gewonnen wurde. So erhält man eine Reihe mikroskopisch sichtbarer, artspezifischer Antwortreaktionen. Normales Kaninchenserum ist unwirksam.

Durch das vergleichende Studium der Aktivität verschiedener Antiseren, sowie durch die Antiserum-Hemmtechnik wurden bis jetzt vier voneinander verschiedene Oberflächenantigene bei den Eiern von *Paracentrotus lividus* festgestellt. Mindestens zwei der gefundenen Antigene haben Polysaccharid-Charakter. Die Reaktionen zwischen einem bestimmten Antikörper und dessen Antigen, das A-Antigen genannt wurde, können interessanterweise zur parthenogenetischen Aktivierung unbefruchteter Eier führen. Andere Antigen-Antikörperreaktionen können Befruchtung und Entwicklung der Eier hemmen. Die mögliche Bedeutung der verschiedenen Antigene für das Festsetzen der Spermien an der Oberfläche der Eier sowie für den Beginn der Ei-Aktivierung wird besprochen.

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Brèves communications - Kurze Mitteilungen
Brevi comunicazioni - Brief Reports

Les auteurs sont seuls responsables des opinions exprimées dans ces communications. – Für die kurzen Mitteilungen ist ausschliesslich der Autor verantwortlich. – Per le brevi comunicazioni è responsabile solo l'autore. – The editors do not hold themselves responsible for the opinions expressed by their correspondents.

Spektraler Farbintegrator

Die uns umgebenden Farben bestehen alle aus spektral gemischtem Licht, dessen Zusammensetzung mit Hilfe der Spektralanalyse in bekannter Weise ermittelt werden kann. Für Zwecke der Farbenlehre ist aber der umgekehrte Vorgang, die Farbsynthese, von ebenso theoretischer wie praktischer Bedeutung.

Farbintegratoren auf trichromatischer Grundlage, die teils mit Farbfiltern, teils mit spektralen Lichtern arbeiten, sind schon verschiedentlich beschrieben worden. Was aber bisher fehlte, ist ein Instrument, das Farben beliebiger spektraler Zusammensetzung in vorgegebener Weise bis an die theoretischen Grenzen hinan zu erzeugen gestattet.

Ein von uns verwendeter Apparat sei hier kurz beschrieben, da er sich uns trotz noch vorhandener Unvollkommenheiten zur Aufklärung und Lösung vieler Farbprobleme als sehr brauchbar erwiesen hat.

Er geht im Prinzip auf eine schon von Newton beschriebene Vorrichtung zurück, wonach ein prismatisch erzeugtes Spektrum auf einer Linse entworfen und von ihr wieder auf einem weissen Schirm zum ursprünglichen farblosen Licht vereinigt wird [1]. Eine analoge Anordnung wurde auch von Pohl [2] und von der Firma Zeiss für Vorlesungszwecke empfohlen. Für unsere besonderen Zwecke waren folgende Bedingungen zu erfüllen:

1. Die verwendete Lichtquelle soll möglichst einer der anerkannten Normlichtarten entsprechen.

[1] J. Newton, *Optik*, Buch I, Teil II, Prop. I, Lehrsatz 1 und Prop. V, Lehrsatz 4 (siehe z. B. «Ostwald's Klassiker», Nr. 96, 1. Buch, pp. 76 und 88).

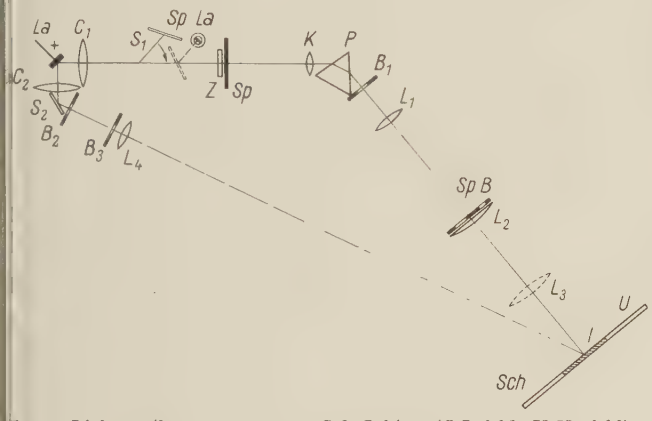
[2] R. W. Pohl, *Optik*, 10. Aufl., p. 338 (Springer-Verlag, 1958).

2. Das in der Farbe beliebig einstellbare runde oder quadratische Feld soll von einem von der Lichtquelle direkt beleuchteten und in der Helligkeit variierbaren Umfeld umschlossen sein.

3. In- und Umfeld sind auf normalweissem Schirm zu entwerfen.

4. Die Grösse des Spektrums ist so zu wählen, dass es nach Zusammensetzung und Intensität mit Hilfe auswechselbarer Blenden mit genügender Genauigkeit messbar verändert werden kann.

5. Ferner soll die Möglichkeit bestehen, filmartige «Rollblenden» zu verwenden, um beliebige Farbreihen wiedergeben zu können.



La	Lichtquelle	*Sch*	Schirm (*I* Infeld, *U* Umfeld)
	Infeldstrahl:		*Umfeldstrahl:*
C_1	Kondensor	C_2	Kondensor
Sp La	Spektrallampe	S_2	Spiegel
S_1	Spiegel (einschwenkbar)	B_2	Umfeldblende zur Begrenzung des Umfeldes und Aussparung des Infeldes
Z	Zylinderlinse		
Sp	Spalt		
K	Kollimatorlinse	B_3	Irisblende zur Regulierung der Helligkeit des Umfeldes
P	Prisma		
B_1	Infeldblende	L_4	Abbildungslinse für B_2
L_1	Abbildungslinse für B_1		
Sp B	Spektralblende (Schiebe- oder Rollblende)		
L_2	Sammellinse		
L_3	Abbildungslinse für *Sp B* (einschwenkbar)		

Als der Normlichtart A, der Farbtemperatur 2850°C, nahestehende Lichtquelle verwenden wir eine Pointolite-Lampe der Siemens Edison Swan Co., London, von 1000 Kerzen, worin die positive Elektrode als kleine viereckige Wolframplatte ausgebildet ist. Die Brenndauer dieser Lampe ist relativ lang, die erforderliche homogene Ausleuchtung des Infeldes, die durch besondere Blendenteste überprüft wird, benötigt aber grosse Sorgfalt. Die Beleuchtungsstärke beträgt auf dem Infeld etwa 75 Lux, die des Umfeldes lässt sich beliebig regulieren.

Die Abbildung gibt unsere Apparatur mit ihren zwei Strahlengängen schematisch wieder.

Unser Instrument kann zum Beispiel zu folgenden Zwecken dienen:

a) Durch stufenweise horizontale Verengerung der Spektralblende lässt sich die ganze Grauskala von Weiss bis Schwarz auf dem normalweissen (Bariumsulfat) Schirm darstellen.

b) Durch Blenden, die bloss ein oder zwei spektrale Sprungstellen zwischen 0 und 1 aufweisen, lassen sich sämtliche Optimalfarben reproduzieren. Bei Wahl dreier optimaler «Grundfarben» kann das Gebiet der damit erzeugbaren Farben leicht überprüft werden.

c) Ist der spektrale Verlauf einer Farbe zum Beispiel mit Hilfe des selbstregistrierenden Instrumentes von Hardy ermittelt, so kann durch entsprechendes Ausschneiden der Spektralblende die Farbe ohne weiteres reproduziert und mit dem Original (im Umfeld) verglichen werden.

d) Natürlich kann auch jede Farbe in Form ihrer Metameren dargestellt werden, wie sie sich z. B. von der Vollfarbe, der Schwarz- oder der Weissoptimalen ableiten lassen[3]. Auch können durch stufenweise horizontale Abblendung der Optimalfarben im Prinzip sämtliche Farbstufen des neuen Deutschen DIN-Farbensystems wiedergegeben werden.

e) Verfertigt man ganze Blendenfolgen und lässt man solche «Rollblenden» ähnlich einem Kinofilm quer zum Spektrum über Walzen laufen, so lassen sich beliebige Farbreihen in kontinuierlicher Folge reproduzieren. Dies erweist sich als besonders vorteilhaft zur Ermittlung der 4 Urfarben sowie der «farbkräftigsten» Farbe im Sinne der kürzlichen Ausführungen von Rometsch, Thürkauf und Miescher[3]. Es ist nun möglich, auch die auf anderem Wege unerreichbaren farbkräftigsten Optimalfarben zu bestimmen.

f) Durch verschiedene Einstellung des Umfangs und der Helligkeit des Umfeldes kann dessen Einfluss auf das Infeld messbar verfolgt werden.

Über unsere Ergebnisse werden wir demnächst ausführlicher berichten.

Zweifellos wird ein derartiges Instrument für alle Zwecke der Farbforschung und Farbgebung in Zukunft eine bedeutsame Rolle spielen. Einstweilen fehlt aber noch eine brauchbare Lichtquelle von genügender Lichtstärke, welche der im Vordergrund stehenden Normlichtart C entspricht. Hochdruck-Xenonlicht kommt ihr am nächsten; doch ist es schwierig, damit ein Infeld von genügender Homogenität zu erzielen.

Spektrale Farbintegratoren auf subjektiver Basis sind schon von Maxwell[4], von Ostwald[5] wie von Rösch[6] beschrieben worden. Sie beruhen auf dem Prinzip des umgekehrten Strahlenganges und stellen natürlich viel geringere Anforderungen an die erforderliche Lichtstärke. Wichtig wird es auch hier sein, das farbige Infeld mit einem (farblosen) Umfeld zu umgeben. Hierüber werden wir später berichten.

M. Gasser, H. Bilger,
K.-D. Hofmann und K. Miescher

Physikalische Anstalt der Universität Basel, 21. November 1958.

Summary

Development of a spectral colour integrator enabling to reproduce on a screen any colour up to the theoretical limits and to determine quantitatively important notions of colour metric.

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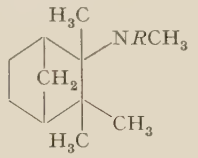
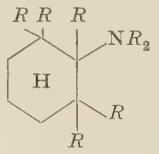
Polymethylcyclohexylamine - neue, hypotensiv wirksame Substanzen[1]

Nach Feststellung der hohen ganglioplegischen und hypotensiven Wirksamkeit von Mecamylamin (I)[2] und Dimecamin (II)[3] hielten wir es für zweckmässig, die Suche nach Substanzen mit ähnlicher Wirksamkeit auf die Gruppe der Polymethylcyclohexylamine auszudehnen, die sich von den Substanzen I und II durch das Fehlen der Methylenbrücke unterscheiden, hingegen mit ihnen die Anhäufung der Methylgruppen in unmittelbarer Nähe des Stickstoffatoms gemeinsam haben. Die Richtigkeit dieses Vorgehens wurde durch kürzlich erschienene Mitteilungen[4,5] über die analoge Wirksamkeit des 1,2,2,6,6-Pentamethylpiperidins (Pempidins) angedeutet, welche die vorläufige Veröffentlichung unserer Ergebnisse beschleunigten.



I, $R = H$
II, $R = CH_3$



III

Die von uns synthetisierten Substanzen können durch die allgemeine Formel III erfasst werden, wobei R Wasserstoffatome oder Methylgruppen darstellen. Sie wurden aus Cyclohexanon, 2-Methyl-, 2,2-Dimethyl-, 2,2,6-Trimethyl- und 2,2,6,6-Tetramethylcyclohexanonen[6,7] dargestellt, und zwar a) durch Reduktion der Oxime mit Natrium und Alkohol (primäre Amine), b) durch Umsetzung mit Methylmagnesiumjodid, Dehydratation, Rittersche Reaktion mit Cyanwasserstoff[8] und nachfolgende Hydrolyse (primäre Amine), c) durch Reduktion der Formamidoderivate mit Lithiumaluminiumhydrid (sekundäre Amine) und d) durch Methylierung der primären Amine mit Formaldehyd und Ameisensäure (tertiäre Amine).

Die von uns dargestellten Substanzen sind in der Tabelle übersichtlich zusammengestellt, wo auch die Schmelzpunkte der Hydrochloride und die vorläufigen Ergebnisse der Bewertung der hypotensiven und ganglioplegischen Wirksamkeit angeführt sind.

Der Einfluss auf den Blutdruck bei der narkotisierten Katze (Chloralose mit Phenobarbital) wird als maximale Druckänderung nach intravenöser Verabreichung von 5 mg/kg der zu untersuchenden Substanz ausgedrückt. Die Blutdruckänderung gegenüber dem Ausgangswert (80–110 mm Hg) um 0–10 mm Hg wird als 0 bewertet, die Senkung um 10–20 mm Hg als −, Anstieg +, um 20–40 mm Hg als −− bzw. ++, über 40 mm Hg als −−− oder +++. Die ganglioplegische Wirkung, die durch die Veränderung der Kontraktionen der präganglionär gereizten Katzennickhaut bewertet wurde, wird bei der Dämpfung der Kontraktionen um 5–25% als −, um 25–50% als −−, um 50–75% als −−− und um 75–100% als −−−− ausgedrückt.

Aus der Tabelle geht hervor, dass einige Polymethylcyclohexylamine ähnliche hypotensive und ganglioplegische Wirksamkeit wie Mecamylamin (I) und Dimecamin (II) besitzen. Von ihnen ist besonders das N,N,1,2,2-Pentamethylcyclohexylamin («Penhexamin») gegenwärtig der Gegenstand unserer eingehenderen Untersuchung.

M. Protiva, M. Rajšner, V. Trčka, M. Vaněček und Z. J. Vejdělek

Forschungsinstitut für Pharmazie und Biochemie, Prag, 1. Dezember 1958.

Hydrochloride der Polymethylcyclohexylamine und ihre Eigenschaften

Substanz Nr.	Cyclohexylamin	Smp. °C	Blutdruckwirkung intravenös (5 mg/kg i. v.)	Ganglioplegische Wirkung intravenös (5 mg/kg i. v.)
III/2862	N,1-Dimethyl	145–146	++	−−−[a,b]
III/2908	*cis*-N,2-Dimethyl	150	0	0
III/2904	*trans*-N,2-Dimethyl	147	0	−
III/2928	2,2-Dimethyl	276	−	−−
III/2868	N,N,1-Trimethyl[9]	183	+	−−−−
III/2903	*cis*-N,N,2-Trimethyl	180–181	+	−−
III/2895	*trans*-N,N,2-Trimethyl	153–154	−	0[b]
III/2944	N,2,2-Trimethyl	213	−−	−
III/2962	1,2,2-Trimethyl	>330	−−	−−−−
III/2943	2,2,6-Trimethyl	265	0	0
III/2934	N,N,2,2-Tetramethyl	180–181	−−	−−
III/2951	N,1,2,2-Tetramethyl	218–219	−	−−−−
III/2917	2,2,6,6-Tetramethyl	>300	−	−−[a]
III/2961	N,N,1,2,2-Pentamethyl	hygroskopisch	−−	−−−−
III/2959	N,N,2,2,6-Pentamethyl	hygroskopisch	−	−−−
	Mecamylamin-HCl (I-HCl)		−−	−−−−
	Dimecamin-HBr (II-HBr)		−−	−−−−

[a] Kurzfristige (vorübergehende) Wirkung. [b] Trotz deutlicher Dämpfung der Kontraktionen steigt der Gesamttonus der Nickhaut an.

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Summary

A series of cyclohexylamine derivatives methylated both on nitrogen atom and in position 1, 2 and 6 of the cyclohexane ring have been prepared. Some of them showed considerable hypotensive and ganglioplegic activity. The most interesting compound is N, N, 1, 2, 2-pentamethylcyclohexylamine ('penhexamine') whose activity compares favourably with mecamylamine or dimecamine.

Albumin Synthesis in Regenerating Rat Liver Cells[1]

In a previous report dealing with the effects of the partial hepatectomy on the rat liver soluble proteins (Guidotti and Clerici[2]), evidence was given that these proteins from the regenerating liver present essentially the same electrophoretic pattern as those from the resting one. However, no reliable data on the albumin behaviour were obtained, because our technique failed to detect this protein.

Since the liver is the site of production of albumin in the rat (Miller *et al.*[3,4], Jensen and Tarver[5]), whose albumin concentration in serum is claimed to control liver regeneration (Glinos and Gey[6], Glinos[7]), an attempt has now been made to investigate the *in vitro* turnover of the above-mentioned protein in the resting and regenerating rat liver.

Male albino rats, Wistar strain, fed a standard diet, were hepatectomized according to Higgins and Anderson[8]. After 12 h fasting, normal and operated animals (72 h after hepatectomy) were killed and their livers perfused with saline solution in order to remove completely the blood proteins[2]. Livers were then taken out, sliced, and pooled to obtain 12 g of wet tissue slices. 6 g of tissue slices were added to a ml 100 flask containing 20 ml of a cold Ringer medium[9], and the whole content of the flask was quickly homogenized and lyophilized. The remaining 6 g of tissue slices were added to a second flask containing 20 ml of the above-mentioned medium supplemented with 20 μM of glycine-1-^{14}C (specific activity 1 $\mu c/\mu M$). After 2 h of incubation in air at 38°C, the flask was cooled in a ice bath; the content was then homogenized and lyophilized as indicated above.

Soluble proteins were extracted according to the technique of Adjutantis[10] as modified by Ciaranfi and Di Sabato[11]. 0·02 and 0·04 ml of a 7% water solution of proteins from each sample, and 0·02 ml of normal rat serum, were utilized for a paper electrophoresis carried out as described by the above mentioned authors[10,11]. All the steps of this procedure were performed in a cold room (+ 2°C).

Amidoschwarz-stained strips were photometrically scanned by means of an Elphor photometer, and the percentage of the protein content was evaluated by planimetry. The albumin-carrying section of each strip was separated from the other soluble proteins and hydrolized with HCl 5 *N* in sealed ampulles kept at 116°C for 16 h. The cloudy suspension obtained thereafter was centrifuged; the supernatant fluid, poured into suitable polythene planchets, was dried under a current of warm air. Radioactivity measurements were performed by means of a windowless flow counter; counts were corrected for self absorption.

After incubation, a protein peak, whose migration rate was similar to that of the serum albumin, was always detected in both the electrophoretic patterns from resting and regenerating rat liver slices. Therefore, this peak, constantly lacking in the electrophoretic patterns from non-incubated liver slices[2], was tentatively identified with albumin, newly released.

As for the evaluation of the amount of albumin released during incubation, some errors may arise from the following causes: (1) protein loss during the extraction procedure; (2) inaccuracy of photometric readings due to different affinity of the several proteins towards Amidoschwarz stain; (3) uncertain separation of the overlapping planimetric peaks, and (4) contamination of the albumin peak from α_1-globulin. It has been estimated, however by experience, that with reasonable care these errors do not exceed $\pm$ 10–15% of the found values. Radioactivity measurements, which were referred to a mg of albumin, are more reliable because not biased by the main error source, i.e. by number (1).

The results concerning the amount of albumin released during incubation and the incorporation rate of the labelled glycine into the same protein are presented in the Table.

It appears that, during incubation, less albumin is released from the regenerating rat liver, as compared to the resting one. However, the difference is not statistically significant ($P > 0{\cdot}05$).

Furthermore, the glycine incorporation rate into the albumin released from the regenerating liver significantly increases over the resting one ($P < 0{\cdot}01$).

[1] This investigation was aided by a grant from C.N.R., Roma, and was made during a tenure by one of us (G.G.) of a Scholarship from C.N.R., Roma.

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[11] E. Ciaranfi and G. Di Sabato, Pubbl. Staz. zool. Napoli *28*, 296 (1956).

Table

Type of tissue used	Number of experiments	Albumin release (mg/g of wet tissue/h)	Glycine-1-^{14}C incorporation into albumin (counts/min/mg)
Resting liver	7	0·23 $\pm$ 0·04*	23·8 $\pm$ 3·0
Regenerating liver	6	0·18 $\pm$ 0·01	55·3 $\pm$ 4·6

* Mean $\pm$ S.E.

This behaviour may suggest that the albumin turnover in the regenerating liver is faster than in the resting one and that the partial hepatectomy, as far as the albumin synthesis is concerned, does not impair the liver function.

The above results rule out the assumptions[12] which attribute the lowering of the serum albumin levels in the hepatectomized animals to a decreased production rate of albumin from the liver cells. On the contrary, they may suggest that the serum albumin concentration decreases after operation because of an increased rate of serum albumin utilization by the liver itself, in order to rebuild its surgically removed parenchima.

G. Guidotti, E. Clerici, G. Sambo, and E. Bazzano

Institute of General Pathology, University of Milan (Italy), September 29, 1958.

Riassunto

Gli autori hanno studiato *in vitro* il problema della sintesi dell'albumina in sezioni di fegato normale e rigenerante di ratto. La quantità di albumina prodotta durante l'incubazione non presenta notevoli variazioni nelle due situazioni studiate. Al contrario l'incorporazione di glicina marcata nella albumina del fegato rigenerante risulta nettamente aumentata rispetto a quella del fegato normale. Tale comportamento viene interpretato come espressione di un ricambio albuminico più elevato nel fegato rigenerante che nel normale.

[12] N. H. Martin and A. Neuberger, Brit. med. Bull. *13*, 113 (1957).

The Local Oedema-Producing Effect of Disodium Ethylendiamine Tetraacetate (EDTA-Na_2)

In a series of experiments Chambers *et al.*[1], and Zweifach[2] found that one of the substances essential to the functional and structural stability of the capillary wall was calcium present in the interendothelial cement substance. Since, at the pH of the organism, calcium is bound by EDTA-Na_2 more effectively than by sodium citrate, it seemed worth while to study the effect of EDTA-Na_2 on capillaries.

Albino rats were injected subcutaneously with a single dose of EDTA-Na_2 *suspension* (60 mg) in the interscapular region. After 7 to 12 h a local, tumor-like oedema developed at the site of injection (Fig. 1). Histologically it showed oedematous structure, poor in cells. Suspensions of sodium citrate (200 mg), sodium acetate (200–400 mg), and pulverised gypsum (200 mg) failed to produce oedema.

The oedema-producing effect of EDTA-Na_2 was also examined with the more sensitive method of Rowley and Benditt[3], by which the severity of the oedema could be measured quantitatively. The oedema produced by a substance injected subcutaneously into the dorsa of the hind paws of rats was demonstrated by intravenously injected Evans blue, which stained the oedematous tissues blue. For details see Ref. [3].

[1] R. Chambers *et al.*, Acta Unio intern. contra Cancrum *6*, 696 (1949).

[2] B. W. Zweifach, Connective Tiss. Conf. Fifth Trans. (J. Macy Jr. Found., New York 1954), p. 42.

[3] K. Rowley and E. Benditt, J. exp. Med. *103*, 399 (1956).

Figure 2 shows that a *solution* containing 370 γ of EDTA-Na_2 or 200 γ of histamine induces practically the same degree of blue colouring, i.e., the two substances are similarly effective in producing oedema. Ca-EDTA-Na_2, on the other hand, not being able to bind calcium, proved ineffective. Figure 3 shows the quantitative data obtained in the course of the experiment. Histamine and EDTA-Na_2 are nearly similar in their effects, whereas Ca-EDTA-Na_2 (410 γ), or sodium citrate (200 γ) do not produce oedema. The antihistaminic agents Antistin and Synopen, or Dibenamine, which is a 5-hydroxytryptamine antagonist[4], do not inhibit oedema produced by EDTA-Na_2.

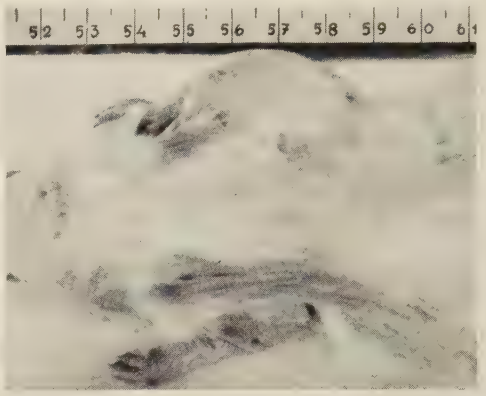


Fig. 1.—Local oedema developed 1 h after the subcutaneous injection of 60 mg of an EDTA-Na_2 *suspension* (in 1 ml of saline)

Discussion. According to our observations subcutaneously injected EDTA-Na_2 produces oedema at the site of injection. The high potency of *suspensions* of EDTA-Na_2 is due to its poor solubility at the pH of the organism resulting in a prolongation of the effect. Since the removal of calcium from the capillary wall increases permeability[2], the oedema-producing property of EDTA-Na_2, which is essentially greater than that of sodium citrate, might be

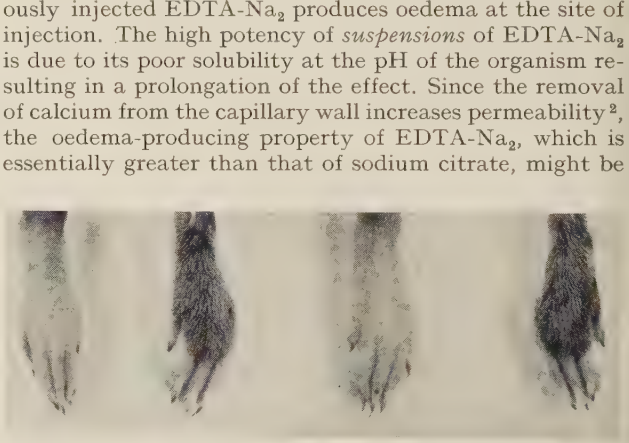


Fig. 2.—Blueing of rat paw skin 2 h after the injection of EDTA-Na_2 (370 γ), histamine (200 γ), and Ca-EDTA-Na_2 (410 γ), in 0·1 ml saline, respectively. Control: 0·1 ml saline

due to a more stable binding of calcium. The ineffectiveness of Antistin, Synopen, and Dibenamine to inhibit EDTA-Na_2-produced oedema shows the direct effect of EDTA-Na_2. The fact that Ca-EDTA-Na_2 is not an oedema-producing substance may be interpreted to mean that not the EDTA-Na_2 molecule *per se*, but its calcium-binding capacity is responsible for the oedema. According to Chambers and Zweifach[5], magnesium, which is also

[4] J. H. Gaddum and K. A. Hameed, J. Pharmacol. *9*, 24 (1954).

[5] R. Chambers and B. W. Zweifach, J. cell. comp. Physiol. *15*, 255 (1940).

bound by EDTA-Na_2, does not play a role in the processes taking place in the capillary wall.

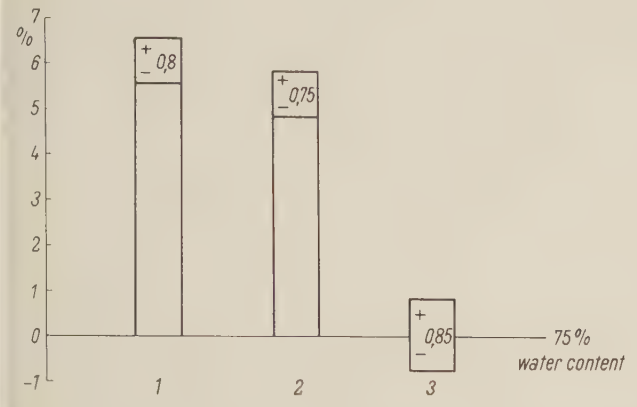


Fig. 3.—The changes in water content of the paw skin of rats. 0% in the diagram indicates the normal water content of skin (75%). Column 1: Histamine (200 γ, subcut.) (6). Column 2: EDTA-Na_2 (370 γ, subcut.) (12), and EDTA-Na_2+Antistin (400 γ, subcut.), or Synopen or Dibenamine (800 γ, intraven.) (18). Column 3: Ca-EDTA-Na_2 (410 γ, subcut.) (6), sodium citrate (200 γ, subcut.) (6) or saline (0·1 ml, subcut.). All substances were dissolved in 0·1 ml saline. The number of animals used in each group is given in brackets.

In 1954 Meyer[6] assumed that EDTA-Na_2 might, in some way, influence capillary function. Our observations would seem to corroborate this concept and suggest that the local oedema-producing effect of EDTA-Na_2 is due to a binding of the calcium in the interendothelial cement substance of the capillaries.

Thanks are due to Dr. A. Körössi and to Dr. S. Sajó, Budapest, for their valuable help and suggestions during the course of this study.

G. Kelényi and J. Kasza

Department of Pathology, Medical University of Pécs (Hungary), September 2, 1958.

Zusammenfassung

Die Lösungen oder Suspensionen von Äthylendiamintetraacetatdinatrium (EDTA-Na_2) erzeugen an der Applikationsstelle Ödeme, welche wahrscheinlich der Bindung von Kalzium in der interendothelialen Zementsubstanz der Kapillaren zuzuschreiben sind.

[6] K. Meyer, Connective Tiss. Conf. Fifth Trans. (J. Macy Jr. Found., New York 1954), p. 73.

The Citric Acid Content of Embryos of *Rana esculenta*

It was previously shown that the mode of determination of the developing crystalline lens in *Rana esculenta* varies with temperature, and that the lactic acid content of gastrulae and neurulae is lower at 12°C than at 25°C[1,2]. Although the effect of temperature on the citric acid content was found to be small, the experimental results may have some value since the literature contains but few data on this subject.

Citric acid content of *Rana esculenta* embryos (µg/100 embryos)

Stage	25°C (*n*)		12°C (*n*)		Average (*n*)	
10	—	—	44	(1)	44	(1)
$10^1/_2$	42	(1)	—	—	42	(1)
11	—	—	46	(1)	46	(1)
$11^1/_2$	—	—	—	—	—	—
12	—	—	—	—	—	—
$12^1/_2$	67	(1)	—	—	67	(1)
13	—	—	—	—	—	—
$13^1/_2$	—	—	—	—	—	—
14	44	(2)	58	(1)	49	(3)
$14^1/_2$	—	—	—	—	—	—
15	—	—	—	—	—	—
$15^1/_2$	—	—	—	—	—	—
16	38	(2)	69	(2)	54	(4)
$16^1/_2$	50	(1)	—	—	50	(1)
17	—	—	50	(1)	50	(1)
$17^1/_2$	56	(2)	68	(1)	60	(3)
18	46	(2)	96	(1)	63	(3)
$18^1/_2$	—	—	—	—	—	—
19	71	(2)	59	(1)	67	(3)
$19^1/_2$	71	(1)	—	—	71	(1)
20	63	(2)	83	(1)	70	(3)
$20^1/_2$	78	(6)	81	(5)	79	(11)
21	75	(4)	73	(3)	74	(7)
$21^1/_2$	—	—	—	—	—	—
22	91	(4)	123	(2)	100	(6)
$22^1/_2$	107	(2)	86	(1)	100	(3)
23	94	(4)	81	(3)	88	(7)
$23^1/_2$	168	(2)	—	—	168	(2)
24	155	(2)	92	(3)	123	(5)
$24^1/_2$	125	(1)	68	(1)	97	(2)
25	128	(7)	67	(3)	111	(10)

Stages according to Shumway.
(*n*) number of duplicate experiments.

Eggs were reared in tap water either at 12°C or at 25°C. For citric acid determinations, 40 to 200 embryos were ground in 10% trichloroacetic acid and after centrifugation the supernatant was assayed by the method of Natelson *et al.*[3]. One extraction was sufficient. Within the range from 10 to 40 µg, the standard deviation was 3%. Mostly the amount of citric acid was calculated per embryo in order to compare identical stages from the same batch, reared at different temperatures. In some cases, fresh or dry weight was determined. Stages were indicated according to Shumway[4].

The results are presented in the Table and Figure 1. The quantity of citric acid per embryo varied largely between different batches, probably mainly because of size differences. However, the general trend is clear: after a slow increase between stages 10 and 17, the content rises more rapidly until stage 22, whereafter it increases still more at 25°C, but declines at 12°C. Finally it decreases at both temperatures. Only in one period, a difference between the two temperature groups could thus be found. In some experiments on larvae, reared at room temperature until some days after stage 25 and fed with vegetal material, dry weight was determined. First, 400 larvae were used for a (duplicate) assay: each larva weighed 0·53 mg and contained 0·71 µg (134 mg%) citric acid. The next day another 400 larvae of the same group, which had been starved for one day, had an average weight of 0·46 mg and contained 0·62 µg (111 mg%). Four experiments were made on ovaries of *Rana temporaria*, shortly before

[1] G. Ten Cate, Nederl. Tijdschr. Geneesk. *90*, 1695 (1946).
[2] G. Ten Cate, Verh. Kon. Nederl. Akad. Wetensch. Amsterdam *51*, Nr. 2 (1956).
[3] S. Natelson, J. B. Pincus, and J. K. Lugovoy, J. biol. Chem. *175*, 745 (1948).
[4] W. Shumway, Anat. Rec. *78*, 139 (1940).

the laying season; these contained 18 mg% citric acid (fresh weight). It may be concluded that citric acid is synthesized especially in the period between stages 17 (tailbud) and 24, when it increases by nearly 200%.

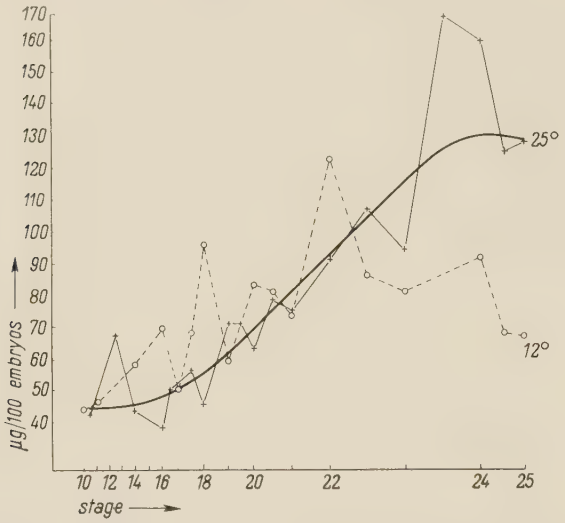


Fig. 1.—Citric acid content of *Rana esculenta* embryos during development at 12°C (broken line) and 25°C. Stages according to Shumway, for *Rana pipiens*

Thereafter it decreases to 0·7 µg per larva, only about 50% more than in the early stages. An effect of temperature is found only between stages 22 and 25, when at 12°C the content remains lower and decreases sooner and more strongly than at 25°C. This might be explained by a difference in the intrinsic, chemical development, in that the cold larvae have reached the lower values of later stages relatively earlier than the warm larvae. Another explanation might be that the metabolism is relatively higher in the cold group, leading to a more rapid exhaustion of the food reserve in the yolk, since the animals were starved. It has been reported that in this species the total amount of oxygen, consumed up to a given larval stage, is about 35% higher at 12 than at 25°C[2]. However, starvation cannot be the sole cause of the decline, since larvae which received plenty of food contained still lower amounts in later stages. A similar decrease was observed by Dickens[5] in the chick, from about 30 mg% in $3^1/_2$- and 5-day old embryos to 13 mg% at 8 and 12 days of incubation. The citric acid content of frog's ovaries (18 mg%) does not differ much from the values found in the yolk of chicken eggs (15·5 and 13 mg%) by Thunberg[6] and by Täufel and Pahloudek-Fabini[7].

For a comparison of the other data with those of the literature, calculations must be made on a fresh weight basis. Previously a dry to wet weight ratio of 1:4 was found in embryos of the same species[2]. Assuming a wet weight of the larvae of 2 mg, and, for this approximate calculation, neglecting the losses due to combustion, the following values may be used: starting development with a citric acid content of 20 mg%, a maximum of 75 mg% is reached, followed by a decrease to 35 mg%. These values are generally higher than those which Dickens found in chick embryos. As compared with animal tissues, the embryonic content is rather high, equalling that of cartilage, in which Dickens found 35 mg%[5]. It must be concluded that the higher values, encountered in tailbud and early larval stages represent a real embryonic citric acid synthesis.

Some possible explanations for this phenomenon may be indicated. First, as indicated by Dickens, it might be a consequence of a high carbohydrate metabolism. However, this type of metabolism prevails only in gastrulae and neurulae. Second, it might be a consequence of a high activity of the citric acid cycle which, through oxidative phosphorylation, can provide a high amount of energy in a period of development when the synthesis of specific chemical components is prominent. It should be pointed out that the amount of citric acid actually present only reflects the equilibrium between synthesis and degradation of this acid in the metabolic cycle. Finally, it is known that the citric acid synthesis is mainly located in the mitochondria (Schneider *et al.*[8]; Székely[9]). The development and differentiation of mitochondria may thus be highly important. Indeed there is an increase both in number and in complexity of these organellas during neurula and tailbud stages (Boell and Weber[10]; Eakin and Lehmann[11]). This might be reflected in the rise of citric acid content. The connections between mitochondria and oxidative metabolism seem to make it worth while to compare the curves of respiratory rate and of citric acid during development. Figure 2 represents both on a semilog scale; the data on respiration (at 25°C) were taken from previous experiments on the same species[2]. Some parallel seems to exist between the third part of the respiratory curve and the citric acid curve. Whether this relation has a real basis must be left open, but it seems to suggest some correlation between both functions of mitochondria *in vivo* experiments. Perhaps the differentiation of mitochondrial crests might be involved in both processes.

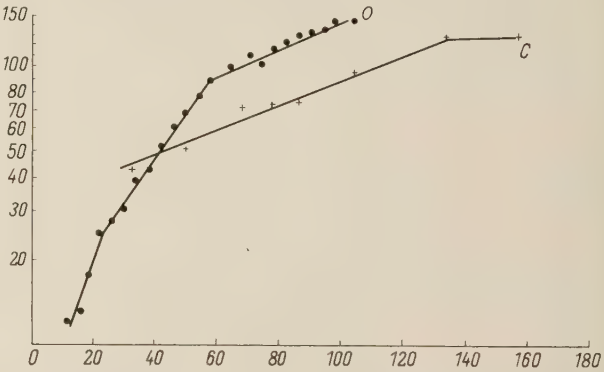


Fig. 2.—Semilogarithmic curves of respiratory rate (*O*) and citric acid content (*C*) of *Rana esculenta* embryos during development at 25°C. Abscissa: time of development. Ordinate: Log. oxygen uptake in µl/h/100 embryos and log. citric acid content in µg/100 embryos

Acknowledgements: Many thanks are due to Mr. C. Marinus, for valuable technical assistance and to Mr. J. Dogger for collecting the material. The work was made possible by a grant from the Netherlands Organization for Pure Research (Z.W.O.).

G. Ten Cate

Division of Chemical Embryology, Laboratory of Anatomy and Embryology, Municipal University, Amsterdam, July 10, 1958.

[5] F. Dickens, Biochem. J. *35*, 1011 (1941).

[6] T. Thunberg, Kungl. fysiogr. Sällsk. Förh. Lund *11*, 126 (1942).

[7] K. Täufel and R. Pohloudek-Fabini, Z. Lebensm. Unters. Forsch. *96*, 397 (1953).

[8] W. C. Schneider, M. J. Striebich, and G. H. Hogeboom, J. biol. Chem. *222*, 969 (1956).

[9] M. Székely, Exper. *13*, 24 (1957).

[10] E. J. Boell and R. Weber, Exp. Cell Res. *9*, 559 (1955).

[11] R. M. Eakin and F. E. Lehmann, Roux' Arch. Entw.-Mech. Organ. *150*, 177 (1957).

Zusammenfassung

Der Zitronensäuregehalt wurde vom Gastrulastadium bis in larvale Stadien gemessen. Während des Schwanzknospenstadiums und kurz nachher erfolgt eine relativ rasche Zunahme, die möglicherweise in Zusammenhang mit der Entwicklung und Differenzierung von Mitochondrien steht.

Influenza del Mg^{++} sulla «eptoformazione non ossidativa» nel muscolo scheletrico

In ricerche precedenti[1–3] abbiamo osservato, in estratti enzimatici di muscolo scheletrico di ratto, il processo indicato da Bonsignore *et al.*[4] col termine di «eptoformazione non ossidativa», e cioè la formazione di eptosofosfato da esoso-fosfato (G-6-P e F-6-P) attraverso reazioni non appartenenti allo shunt ossidativo. Nelle nostre condizioni, tale eptoformazione veniva aumentata per aggiunta di fruttoso-1,6-difosfato (F-1,6-P).

Per spiegare i dati sperimentali abbiamo ammesso – come suggerito da Bonsignore *et al.*[4] – che la velocità del processo eptoformativo dal solo esoso-fosfato fosse «limitata» da tracce di trioso-fosfato presenti nell'estratto enzimatico. Nelle nostre condizioni, il F-1,6-P aggiunto avrebbe agito in quanto aumentava, per scissione aldolasica, la concentrazione del trioso-fosfato.

Nella Tabella seguente sono esposti i risultati di alcune esperienze[5] che suffragano, a nostro parere, questa ipotesi.

Dalla Tabella risulta:

1) Aumentando la concentrazione del F-6-P – nelle prove contenenti questo solo substrato – da 7,5 a 10,0 μM, la eptoformazione non amuenta. Quindi nelle prove enzimatiche in presenza di «F-6-P+F-1,6-P», l'aumento della concentrazione del F-6-P dovuto alla idrolisi del legame fosforico in 1 dell'estere difosforico, non dovrebbe influire sulla eptoformazione, essendo già ottimale la concentrazione delle 7,5 μM di F-6-P aggiunte.

2) Aggiungendo Mg^{++}, attivatore della idrolisi fosfatasica in 1 a carico del F-1,6-P, si ha – nelle prove in presenza di ambedue i substrati – una diminuzione della eptoformazione. Questa diminuzione è spiegabile in quanto la attivazione dell'idrolisi fosfatasica da parte degli Mg^{++} sottrarebbe il F-1,6-P alla azione aldolasica. Ne deriverebbe una minore formazione di trioso-fosfato e conseguente minore eptoformazione.

3) L'aggiunta, oltre al Mg^{++}, di KF – inibitore della attivazione degli ioni Mg^{++} sull'attività fosfatasica del nostro estratto – ripristina pressochè quantitativamente l'eptoformazione nelle prove in presenza di F-6-P + F-1,6-P.

Tutti questi fatti depongono a favore dell'ipotesi che il F-1,6-P nelle nostre condizioni agisca come donatore di trioso-fosfato, necessario per la eptoformazione, e non come donatore di F-6-P.

Attività eptoformativa di estratti enzimatici di muscolo scheletrico a partire da F-6-P e da F-1,6-P, in assenza e in presenza di $MgCl_2$ (10 μM) e di $MgCl_2$ (10 μM) + KF (200 μM). Volume finale delle prove: 2,5 ml

Substrati	Aggiunte	μM eptoso formato/ mg N proteico	% F-1,6-P idrolizzato*
7,5 μM F-6-P	—	0,18	—
10 μM F-6-P	—	0,17	—
7,5 μM F-6-P	$MgCl_2$	0,18	—
7,5 μM F-6-P + 1,8 μM F-1,6-P	—	0,41	53%
7,5 μM F-6-P + 1,8 μM F-1,6-P	$MgCl_2$	0,27	62%
7,5 μM F-6-P + 1,8 μM F-1,6-P	$MgCl_2$ + KF	0,36	48%
1,8 μM F-1,6-P . . .	—	0,12	50%
1,8 μM F-1,6-P . . .	$MgCl_2$	0,13	62%

* L'idrolisi del F-1,6-P è stata constatata dosando i fosfati liberi nel mezzo, prima e dopo l'incubazione, con il metodo di Berenblum e Chain[6]. Tale idrolisi, maggiore in presenza di Mg^{++}, si svolgeva a livello del legame fosforico in 1. Infatti il F-6-P non veniva idrolizzato nè in assenza nè in presenza di Mg^{++}.
È noto d'altra parte che gli ioni Mg^{++} attivano la F-1,6-P-asi del muscolo scheletrico[7].

V. Moret e S. Sperti

Istituto di Chimica Biologica, Università di Padova, il 3 ottobre 1958.

Summary

Mg^{++} influence on 'non-oxydative heptoformation' from hexose-phosphate has been studied in enzymatic preparations of rat skeletal muscle. The results give further evidence that F-1,6-P, added to F-6-P, increases the rate of heptoformation inasmuch as it gives, by aldolase action, triosephosphate.

[1] V. Moret e S. Sperti, Exper. *14*, 342 (1958).
[2] S. Sperti e V. Moret, Exper. *14*, 358 (1958).
[3] S. Sperti e V. Moret, Boll. Soc. ital. Biol. sper. (1958) in corso di stampa.
[4] A. Bonsignore, S. Pontremoli, G. Fornaini e E. Grazi, G. Biochim. *6*, 241 (1957).
[5] Il metodo di preparazione dell'estratto, le condizioni sperimentali seguite nelle prove enzimatiche, ed i metodi di dosaggio sono esposti in una nota precedente [1].
[6] I. Berenblum e E. Chain, Biochem. J. *32*, 295 (1938).
[7] K. Lohmann, Biochem. Z. *262*, 137 (1933).

Analyses immunoélectrophorétiques des fractions protéiques du sérum humain séparées par électrophorèse en gel d'amidon

Le sérum humain, soumis à une électrophorèse en gel d'amidon se scinde en de nombreux composants (Smithies[1]). Ce pouvoir de résolution du milieu, plus élevé que celui du papier ou de la gélose résulte d'un phénomène complexe régissant dans un gel d'amidon, la position des fractions en fin d'électrophorèse.

Dans les modes habituels d'électrophorèse de zone (papier ou gélose) la mobilité en fin d'expérience est due à la résultante du courant électrique et de l'électro-osmose. En gel d'amidon la mobilité habituelle d'une protéine se trouve perturbée par le pouvoir du gel à ralentir les protéines composées de grosses molécules, à l'avantage de celles composées de molécules de plus petites dimensions.

[1] O. Smithies, Biochem. J. *61*, 629 (1955).

C'est ainsi qu'une fraction homogène à l'électrophorèse sur papier ou en gélose se scinde en plusieurs composants si sa population comprend des molécules de dimensions variées. On comprend alors que les globulines α^2 par exemple se trouvent scindées en gel d'amidon en plusieurs composants, les uns en avant des β-globulines; les autres en arrière de celles-ci.

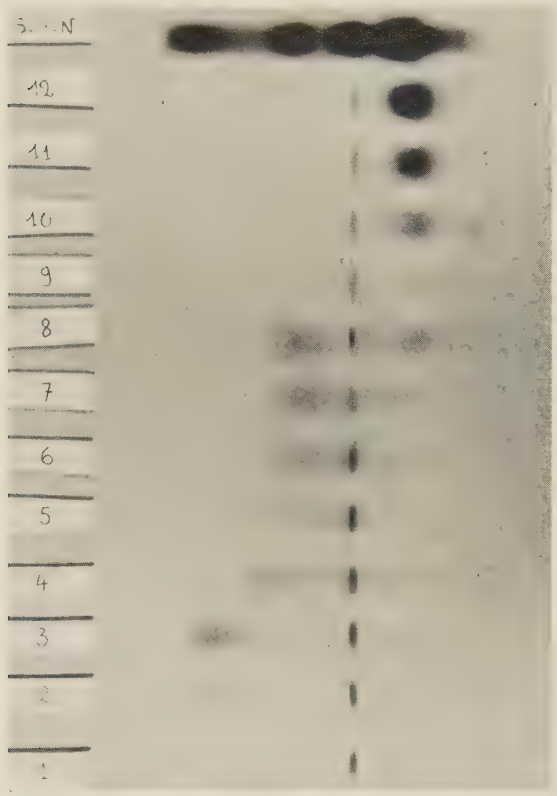


Fig. 1. Electrophorèse en gélose des éluats obtenues à partir des fractions séparées par électrophorèse en gel d'amidon, coloration à l'amidoschwarz

1, 2, 3 = γ-Globulines; 4 = β-lipoprotéines; 5 = α 2 lentes; 6 = α–β; 7 = β-globulines; 8 = α 2 rapides; 9 = postalbumines; 10, 11, 12 = albumines

La nomenclature des différentes fractions protéiques du sérum humain a été établie soit par comparaison avec des fractions obtenues par électrophorèse sur papier, soit à l'aide de l'électrophorèse bidimensionnelle, la première dimension étant réalisée sur papier, et la seconde en gel d'amidon (Poulik et Smithies[2]).

Dans ce travail nous avons étudié par la méthode immuno-électrophorétique de Grabar et Williams[3], la nature immunologique de chaque fraction séparée en gel d'amidon et préalablement éluée.

Protocole expérimental. L'électrophorèse en gel d'amidon est réalisée suivant les modalités que nous avons décrites dans une précédente communication[4]. Après séparation électrophorétique, on coupe sur toute la longueur du gel une tranche supérieure et une tranche inférieure afin d'éliminer les phénomènes de traînées existant en surface. Les faces internes de ces tranches sont colorées au bleu de bromophénol alcoolo-acétique (coloration immédiate). Par référence aux fractions colorées on peut alors découper le gel d'amidon en autant de tranches perpendiculaires que l'on voit de fractions. Chaque tranche est additionnée de 20 ml d'eau physiologique, broyée, centrifugée à 10000 T/min 30 min, le surnageant est recueilli; lyophilisé, et repris par 1 ml d'eau distillée. Chaque éluat est ensuite soumis à une immunoélectrophorèse pratiquée en gélose avec du sérum de cheval antisérum humain préparé par P. Grabar[5].

Résultats. La méthode immunoélectrophorétique, par sa sensibilité permet de révéler la nature antigénique des éluats obtenus.

Les fractions albumine, postalbumines, β-lipoprotéines et γ-globulines obtenues par électrophorèse en gel d'amidon se présentent comme des fractions immunologiquement homogènes, ne donnant qu'une seule ligne de précipitation avec le sérum antihumain (voir Figure 2). Leur nature immunologique confirme entièrement l'appellation qui leur a été donnée par Smithies. Les autres fractions par contre apparaissent comme immunologiquement hétérogènes.

Les globulines α 2 rapides (F α_2) donnent une ligne de précipitation de mobilité α 1, une α 2 et une β 1. Les globulines α 2 lentes (S α_2) donnent une ligne de mobilité α 2 et une ligne β 1, enfin les globulines β, une ligne β 1 et une ligne α 2. Dans certaines expériences, ces globulines β ne donnent qu'une ligne β 1.

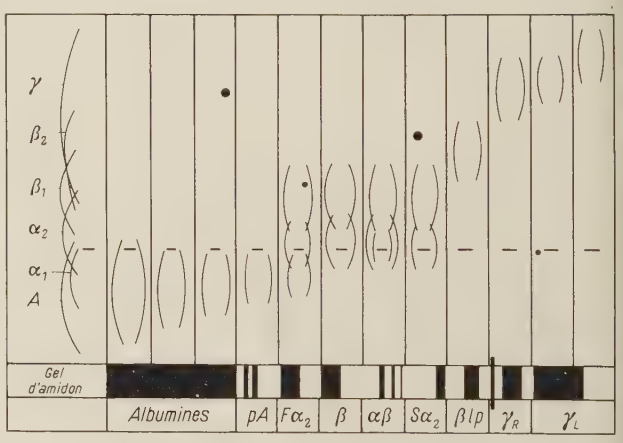


Fig. 2. Immunoélectrophorèses en gélose des mêmes fractions. Sérum de cheval antisérum humain (Institut Pasteur, Paris)

Discussion. L'hétérogénéité immunologique de ces fractions peut trouver une explication dans l'hypothèse suivante:

Sur un fond de protéines constituant une véritable solution de continuité se détachent des fractions qui nous apparaissent comme nettement délimitées. L'analyse immunoélectrophorétique de ces fractions montre que celles-ci sont constituées d'un mélange en proportions inégales d'un constituant principal et de constituants qui sont ses voisins immédiats, et ayant une mobilité soit légèrement plus rapide, soit légèrement plus lente que le constituant principal.

A l'appui de cette théorie, nous pouvons citer le fait que, si l'on soumet un sérum à une plus grande différence de potentiel pendant une durée d'électrophorèse plus longue, on voit apparaître un nombre plus grand de

[2] M. D. Poulik et O. Smithies, Biochem. J. *68*, 636 (1958).

[3] P. Grabar et C. H. Williams, Biochem. biophys. Acta *10*, 193 (1953); *17*, 67 (1955).

[4] J. M. Fine, J. Loeb et C. Waszczenko-Z, Nature *182*, 452 (1958).

[5] Sérum commercialisé par l'Institut Pasteur, Paris.

constituants, ceux-ci sont issus de cette solution de continuité et sont alors rassemblés sous forme de nouvelles bandes.

Conclusion. L'obtention par élution de fractions parfaitement homogènes après électrophorèse en gel d'amidon, est extrêmement difficile et doit être soumise comme contrôle de pureté à l'analyse immunoélectrophorétique, capable par sa sensibilité de révèler éventuellement l'hétérogénéité de la fraction envisagée.

J. M. Fine et J. Loeb

Centre National de Transfusion sanguine, Paris, le 3 novembre 1958.

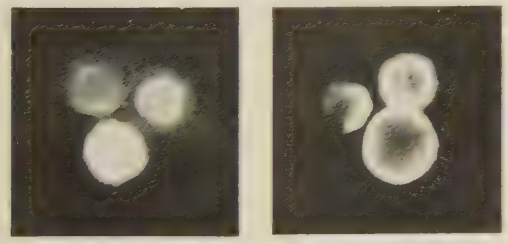
Summary.

Human plasma components, separated by starch gel electrophoresis, are eluted and concentrated by lyophilisation. Immuno-electrophoretic analysis shows that certain of these fractions are immunologically heterogenous.

The authors point out that this immunological criterium should be used before employing an eluted fraction, even if it appears electrophoretically homogenous.

The Demonstration of D (Rh) Antigen in Human Leukocytes

Observations on the presence of D (Rh) antigen in human leukocytes are scarce and contradictory[1,2]. Results described in this communication indicate that D antigen may be detected in white cells from Rh-positive persons by means of the fluorescent antibody technique first described by Coons and Kaplan[3].



A *B*

The staining technique consists of three steps: (1) incubation of leukocytes with anti-D serum; (2) exposure of sensitized white cells to chicken anti-human globulin serum, and (3) staining with anti-chicken globulin fluorescein conjugate (kindly prepared by Dr. A. Prince). The leukocytes from Rh-positive and Rh-negative persons were investigated. The controls were as follows: a washed cells, examined for autofluorescence; (b) leukocytes directly exposed to fluorescein conjugate; (c) sensitized white cells stained with fluorescein conjugate; (d) leukocytes treated with chicken anti-human globulin serum and incubated with conjugate, and (e) white cells from Rh-positive persons incubated in normal human serum which does not contain anti-D antibody. A Reichert fluorescent microscope equipped with a high pressure mercury vapor lamp and dark field condenser was used. The photographs were taken on Agfa Fluorapid Film which is particulary sensitive to yellow-green light.

Typical results are shown in the Figure in *A* the white cells are seen in ordinary dark field, and in *B* with ultraviolet light. Controls are equally visible in ordinary dark field, but did not fluorescence yellow-green. A weak bluish autofluorescence was sometimes visible. Positive results are only obtained with leukocytes from Rh-positive persons. The full report will be published elsewhere.

Mr. T. L. Lincoln is supported by a U.S. Public Health Service Fellowship, and a James Hudson Brown Fellowship, Yale University.

B. D. Janković and T. L. Lincoln

Microbiological Institute, Belgrade University School of Pharmacy, Belgrade (Yugoslavia), September 30, 1958.

Zusammenfassung

Mit indirekter Fluoreszenz-Antikörper-Methode wurde nachgewiesen, dass Leukozyten Rh-positiver Versuchspersonen D-Antigen enthalten.

Immunology of Toxemias of Pregnancy
II. Immunological Reactivity in Eclamptic Conditions

In the first part of this study we have pointed out the presence of autoantibodies in the sera of pregnant women suffering from preeclampsia. In this part we are following immunological reactivity in this disease.

Materials and Method. A group of 86 women whose pregnancy had lasted from 2 to 8 months were each given a single antigenic stimulus by injecting subcutaneously 0·5 ml Brucella bacterine of Nr. 2 density of the McFarland scale. The immunization had been preceded by control tests for antibody to Brucella, with uniformly negative results. The response by antibody was assessed thrice: on the 7th, the 14th and the 21st days following the impulse. Evaluation of immunological reactivity was based solely on findings of incomplete antibodies. In cases where their titre did not exceed the titre of agglutinins, it was presumed that both titres were identical. The method employed had been described in detail in previous papers[1-3].

Only those women whose history was free of brucellosis, allergic diseases, acute rheumatic fever and acute diffuse glomerulonephritis were selected for the research. Simultaneously with the antigenic impulse, they were subjected to a thorough medical and gynaecological investigation; in none of them was the result pathological.

After parturition the women were divided, according to their course of pregnancy following the immunization, into a group with pathological (preeclamptic) course of pregnancy (19 cases), and into a control group (67 cases). Symptoms adopted for the determination of toxemia of

[1] V. Wagner, V. Rejholec, and V. Malý, Ann. rheum. Dis. *14*, 243 (1955).

[2] H. Padovcová, V. Rejholec, V. Malý, F. Suda, and V. Wagner, Ann. Paediatr. *187*, 351 (1956).

[3] V. Rejholec, V. Fencl, V. Wagner, M. Repić-Šlechta, and E. Hallerová, Minerva nefrolog. *2*, 3 (1955).

pregnancy were: Finding of albumin in the urine, increase in blood pressure above 150/100 mg Hg, swellings.

Results. Statistical evaluations did not reveal any association between the age of the women, or between the advance of pregnancy, on the one side and elevation of the antibody titre, or the incidence of toxemia on the other. This made it possible to treat each of the groups as a whole while comparing their immunological reactivity.

Fig. 1

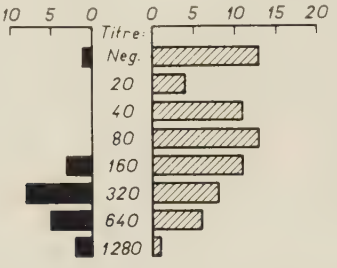


Fig. 2

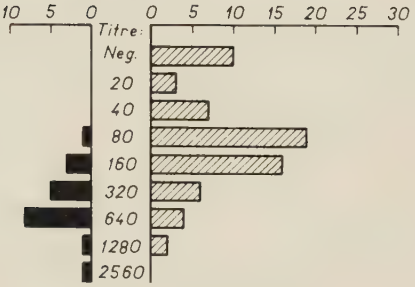
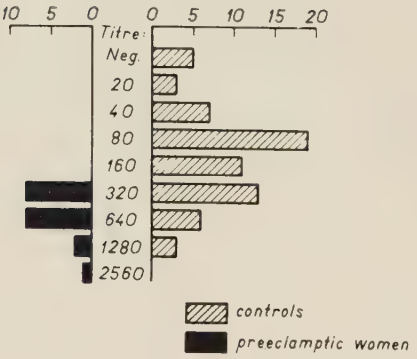


Fig. 3



It was found that already on the 7th day after the antigenic impulse the titres of incomplete antibody to Brucella antigen were statistically significantly higher in the preeclamptic group than in the control group ($P = 0{\cdot}05$). This difference still increased on the 14th and the 21st day and became higly significant ($P = 0{\cdot}01$). Figure 1 and 2 show the result of the titration of incomplete antibody on the 14th and the 21st days following immunization, and Figure 3 brings a comparison of all individual top titre levels irrespective of the time elapsed since immunization. All these graphs, but especially clearly the last one, bring to light the elevation of incomplete antibody titres in the group of preeclamptic women in comparison with the controls.

Discussion. The increase in the ability to produce incomplete antibody has already repeatedly been demonstrated for acute rheumatic fever[1,2], and acute glomerulonephritis[3]. The observations which are reported here suggest that preeclamptic conditions may also be classed with the diseases characterized by an increased immunological reactivity. On the other hand, our results do not reveal the mechanism by which hyperreactivity predisposes to pathological developments. It is true that in the group of preeclamptic women there was not a single case in which incomplete antibody to Brucella antigen was not present in a relatively high titre. Inversely, there were several pregnant women in whom signs of preeclampsia were lacking but who also reacted to the Brucella stimulus with a significant titre. Thus, it may well be that hyperreactivity is only one of the essential preconditions for the genesis of toxemia of pregnancy. Some further factor or complex of factors may be involved in this pathogenesis. For acute rheumatic fever and for acute glomerulonephritis, the etiological role of streptococcus pyogenes has been established: no similar agent has so far been detected for preeclampsia. Possible complicity of the foetus has been suggested by a number of workers but as yet not clearly defined. The auto-immunization processes described may well be conditioned primarily by hyperreactivity, but one should not leave out of account further possible factors, such as the easiness with which the antigenic structure of tissues may change, a certain antigenic affinity of different organs, as well as external influences which may also come into play in antigenic changes in the organism.

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Institute for Microbiology, Medical Clinic, Clinic for Gynecology and Obstetrics of the University of Pilsen and Institute for medical Organization of the Karl-University of Prague, September 3, 1958.

Résumé

Après une seule injection d'un vaccin brucellique on trouve chez les femmes atteintes d'éclampsie une production d'anticorps incomplets significativement plus élevée que chez les femmes dont la grossesse a été normale.

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Maintenance of the Ability of Cells Cultivated *in vitro* to Commence Formation of Antibodies

It has been demonstrated in a number of papers[1] that mesenchymal cells removed from immunized donors continue to produce antibodies in tissue cultures. It is, however, still doubtful whether production of antibodies can be demonstrated in tissue cultures if the tissue is explanted shortly after injection of the antigen, i.e. up to 48 h, during the inductive phase. The majority of experiments in which antigen was added to an explantate of tissue from a non-immunized donor gave negative results[2]. Recently a few positive results have been reported[3].

In experiments performed during recent years, it was shown in our laboratory[4] that spleen tissue explanted

[1] A. Fagraeus, Acta med. scand. *130*, Suppl. 204 (1948). – G. J. Thorbecke and F. J. Keuning, J. Immunol. *70*, 129 (1953); J. infect. Dis. *98*, 157 (1956). – A. B. Stavitsky, J. Immunol. *75*, 214 (1955). – D. Steiner and H. Anker, Proc. nat. Acad. Sci., Wash. *42*, 580 (1956).

[2] R. C. Parker, Science *85*, 292 (1937). – A. J. Salle and W. A. McOmie, J. Immunol. *32*, 157 (1937). – D. Steiner and H. Anker, Proc. nat. Acad. Sci., Wash. *42*, 580 (1956).

[3] W. Höpken, Virchows Arch. *325*, 39 (1954). – K. M. Stevens and J. M. McKenna, Nature *179*, 870 (1957); J. exp. Med. *107*, 537 (1958).

[4] J. Šterzl and M. Rychlíková, Czechoslov. Microbiol. *2*, 334 (1957); Folia biol. *4*, 11 (1958).

shortly after immunization and cultivated in both a natural medium (Tyrode solution and equal part of rabbit serum) and in a synthetic medium (Parker's medium 199 + 0·5% homologous, i.e. rabbit albumin) does not form antibodies. They could also not be demonstrated in a synthetic medium that was further concentrated by pressure dialysis and lyophilization. Cultivation of tissues explanted 24 h after immunization always gave negative results. Explantation of tissues 48 h after immunization gave a positive result only in one out of 11 cases and only after concentration of the cultivation medium. In control experiments in which spleen tissue was explanted and cultivated when antibody formation was fully developed, e.g. after several doses of antigen, antibodies could be demonstrated directly in the medium and the antibody titre increase was proportional to the concentration. Antibodies could never be shown to be present in experiments in which normal explanted tissue was mixed with antigen *Brucella suis in vitro* and cultivated for up to 9 days[5]; not even if the tissue was first stimulated by the *Salmonella paratyphi* B antigen or purified lipopolysaccharide as was done by STEVENS.

Cultivation experiments clearly showed that it is not possible to realise the first inductive phase of antibody formation in tissue cultures. Using only the tissue culture method, it was not possible to decide whether tissue cultures do not produce antibodies because the cellular mechanism of antibody formation is inactivated – as was shown by HARRIS using incubation of cells at 37°C[6] – or whether the inductive phase is more sensitive to unfavourable physical and metabolic conditions in tissue cultures – as to the effect of X-rays[7] and cortisone[8] – or whether cells capable of reacting with the antigen cannot divide mitotically in tissue cultures and differentiate into cells producing antibodies.

We attempted to solve this problem by combining cultivation of spleen cells in tissue cultures with cultivation of cells *in vivo*, i.e. with cell transfer into the peritoneum of newborn rabbits. Infant animals and embryos, although not themselves capable of reacting to an antigen by antibody formation, provide cells with a favourable environment in which they can multiply, differentiate, and commence antibody formation[9].

The experiment was arranged as shown in Figure 1. Isolated spleen cells are mixed with corpuscular or liquid antigen of *Brucella suis*. Part of the cells at a concentration of 60×10^6/ml is transferred into the peritoneum of infant rabbits. Antibody formation by the cells was studied in the serum of the infant animals by agglutination[9]. The remaining part of the cells is transferred to a tissue culture in an Evans apparatus[10] or an Erlenmayer flask (vol. 250 ml, 10 ml cultivation medium). Spleen cells were added to Parker's medium 199[11] + 0·5% rabbit albumin

[5] J. ŠTERZL, Abstracts VII[th] Int. Congr. Microbiol., p. 226 (1958).
[6] S. HARRIS, T. N. HARRIS, and M. B. FARBER, J. Immunol. *72*, 148 (1954).
[7] C. G. CRADDOCK and J. S. LAWRENCE, J. Immunol. *60*, 241 (1948). – H. I. KOHN, J. Immunol. *66*, 525 (1951). – F. J. DIXON, D. W. TALMAGE, and P. H. MAURER, J. Immunol. *68*, 693 (1952). – W. H. TALIAFERRO, L. G. TALIAFERRO, and E. T. JANSSEN, J. infect. Dis. *91*, 105 (1952).
[8] K. BERGLUND and A. FAGRAEUS, Atti VI. Congr. int. microb. *2*, 231 (1953).
[9] J. ŠTERZL, Folia biol. *1*, 193 (1955); *3*, 1 (1957). – J. ŠTERZL and Z. TRNKA, J. hyg. epid. Microbiol. *1*, 292 (1957). – Z. TRNKA, Nature *181*, 55 (1958). – M. HOLUB, Nature *181*, 122 (1958).
[10] V. J. EVANS, W. R. EARLE *et al.*, J. nat. Cancer Inst. *11*, 907 (1951). – D. S. DANES, Exp. Cell Res. *12*, 169 (1957).
[11] J. F. MORGAN, H. J. MORTON, and R. C. PARKER, Proc. Soc. exp. Biol. Med., N. Y. *73*, 1 (1950).

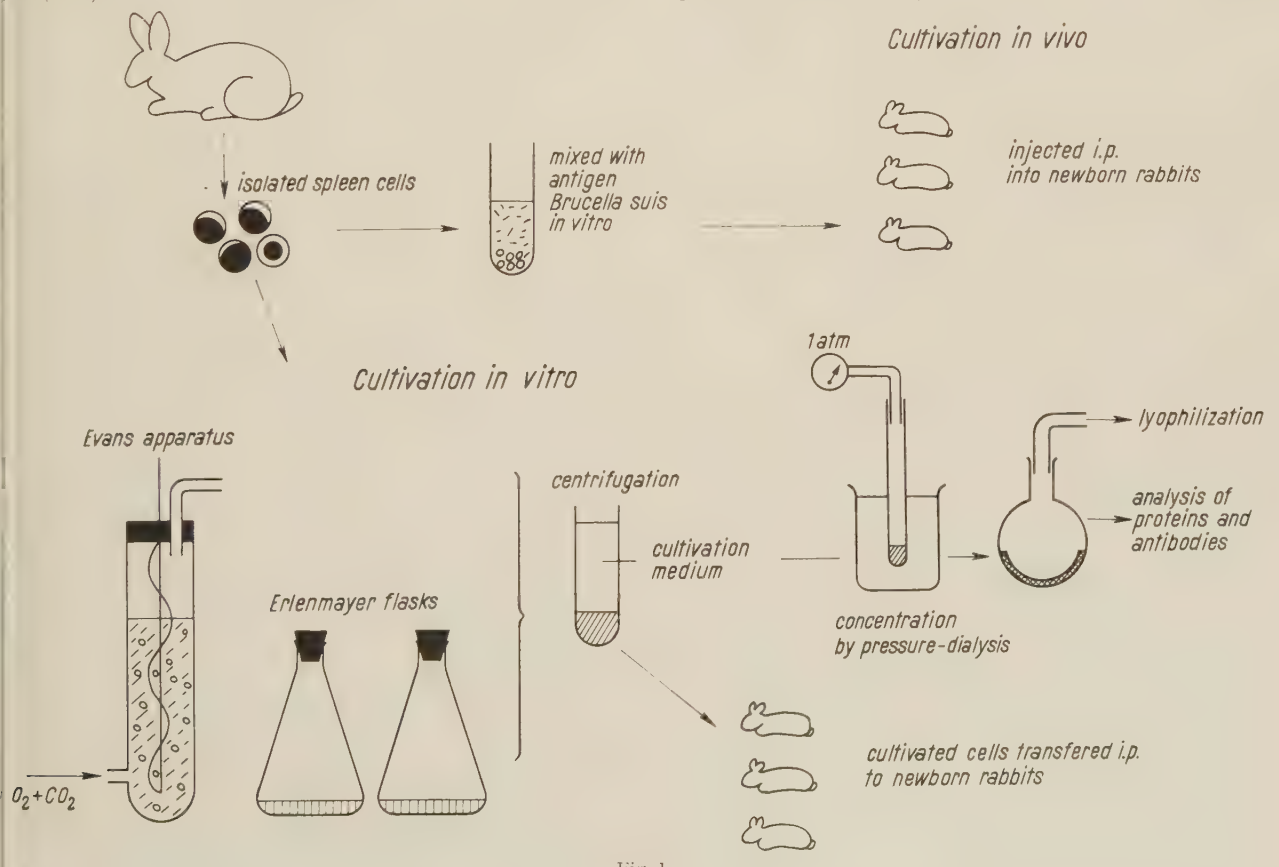


Fig. 1

so that the final cell concentration was 5×10^6/ml medium. They were aerated with 96% O_2 + 4% CO_2 and cultivated at 37°C for 6, 12, 24, 48 h up to 9 days. At the same time, a control series was always started, prepared under the same conditions, and kept at 4°C; 200 ml of medium were used in each experiment. The number of lymphoid cells counted in a Bürcker chamber and the viability test with trypan blue were determined every day of cultivation. After terminating cultivation at the times indicated above, the medium was concentrated and analysed for antibodies using agglutination with *Brucella suis* antigen. γ-Globulin was determined electrophoretically. After centrifugation, the cultivated cells were adjusted to the same concentration as for transfer to infant rabbits, i.e. to 60×10^6 cells/ml. This suspension of cultivated cells was again transferred to newborn rabbits.

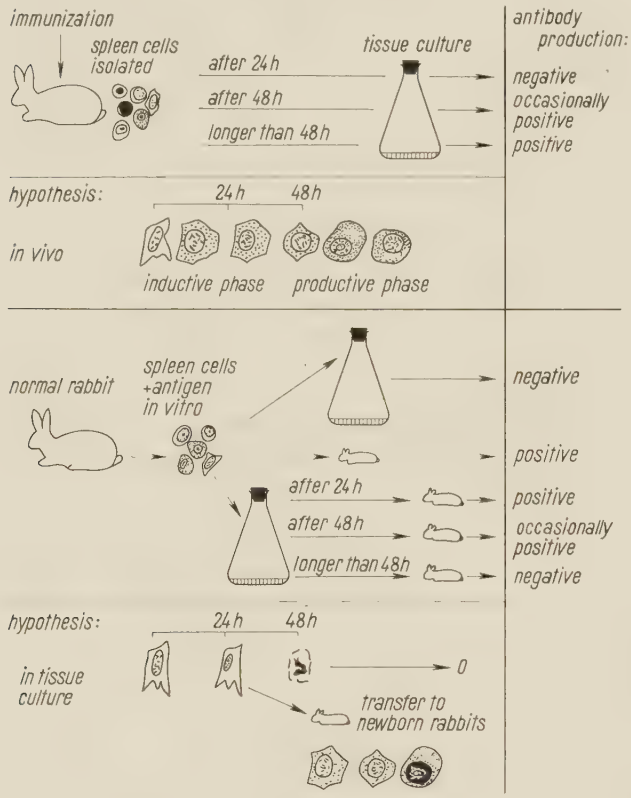


Fig. 2

Antibodies were never found in any experiment with cultivation of cells together with the antigen *in vitro*, not even after concentrating the culture medium 40 times. If, however, the cultivated cells were transferred after 6, 12, 24, and occasionally also 48 h cultivation to infant rabbits, antibody formation was induced (titre 1:32–256). If cells were transferred after longer cultivation (72 h to 9 days) formation of antibodies could never be demonstrated after transfer to infant rabbits.

Figure 2 summarizes the results of cultivation and the conclusions of the experiment. If spleen cells were taken from an immunized donor, antibodies could only be demonstrated if cells already producing antibodies were used for tissue culture. If isolated cells from a non-immunized donor were cultivated with an antigen *in vitro*, antibody formation in the tissue culture could never be demonstrated under any of the experimental conditions. Mixing the same cells *in vitro* with the antigen and subsequently transferring them to infant rabbits always resulted in antibody formation. If these cells were cultivated and transferred after certain time intervals into infant rabbits it was demonstrated that the ability to form antibodies in a tissue culture is maintained for only 24–48 h after explantation; then it disappears.

The results are interpreted to indicate that contact of the antigen with primitive mesenchymal cells[12] and their further division and differentiation[13] are necessary for antibody formation to commence – for the inductive phase. As long as the formation of productive cells is not terminated under conditions permitting these changes, i.e. *in vivo*, formation of antibodies in tissue cultures does not occur. Cells able to commence antibody formation do not lose this ability *in vitro* and survive there for a certain period. Formation of antibodies is only realised if they are transferred to suitable conditions, i.e. when cultivated *in vivo*.

J. Šterzl

Division of Immunology, Institute of Biology, Czechoslovac Academy of Science, Praha, September 20, 1958.

Zusammenfassung

Aus einem normalen, nicht immunisierten Kaninchen isolierte Milzzellen, welche *in vitro* 6, 12, 24 und 48 h kultiviert und nachher zusammen mit *Brucella-suis*-Antigen in das Peritoneum neugeborener Kaninchen übertragen wurden, erzeugen Antikörper. Die Antikörper werden im Serum der Empfänger, das heisst neugeborener Kaninchen, welche selbst noch nicht zur Antikörperbildung befähigt sind, festgestellt. Falls die Milzzellen nach 72stündiger oder längerer Kultivierung auf neugeborene Kaninchen übertragen werden, ist diese Übertragung niemals von Antikörperbildung begleitet.

[12] J. Šterzl, *Mesenchymal tissue during immunisation and infection* (Praha 1954).

[13] R. W. Wissler, F. W. Fitch, M. F. LaVia, and C. A. Gunderson, J. cell. comp. Physiol. *50*, Suppl. 1, 265 (1957).

Meccanismo dell'azione attivatrice esplicata dal Guanosintrifosfato (G.T.P.) sui sistemi enzimatici ossidasici dei grassi. Nota IV

I sistemi enzimatici ossidasici dei grassi preparati dal fegato di cavia sono attivati dall'A.T.P., dal G. T. P. e dall'α-chetoglutarato[1–3]. Il G. T. P. e l'α-chetoglutarato sembrano agire con meccanismo analogo, diverso da quello esplicato dall'A. T. P. Poichè il processo di attivazione consiste nel fornire energia al sistema enzimatico ossidasico dei grassi per la sintesi degli acil-Coenzima A – sintesi indispensabile affinchè il processo ossidativo possa svolgersi – si può prospettare che tale energia possa essere fornita dal G. T. P. e dall'α-chetoglutarico con due meccanismi: 1) o indirettamente nel senso che sia il G.T.P. che l'α-chetoglutarato forniscono energia per la sintesi del succinil-CoA secondo le seguenti reazioni *a*) e *b*):

a) G. T. P. + acido succinico + CoA = succinil-CoA + G. D. P. + Pi;

b) α-chetoglutarato + CoA $\xrightarrow[+\,1/2\ O_2 - CO_2]{}$ succinil-CoA

e a sua volta il succinil-CoA potrebbe trasferire il CoA direttamente all'acido grasso secondo la seguente reazione *c*):

[1] M. Sacchetto e C. R. Rossi, Exper. *14*, 253 (1958).

[2] C. R. Rossi e M. Sacchetto, Exper. *14*, 254 (1958).

[3] M. Sacchetto e C. R. Rossi, Exper., in corso di pubblicazione.

μM O_2 consumate per l'ossidazione dell'acido caprilico nello spazio di tempo di 20 min da 100 mg di estratto enzimatico secco. Caprilato $0{,}7 \times 10^{-3}$ *M*. α-chetoglutarato 1×10^{-3} *M*. Mg^{++} 1×10^{-2} *M*. G. T. P. 10 μM. AsO_4^{---} 40 μM. Tampone Na–K pH 7,4 cm^3 2,4. Preparato enzimatico secondo Lehninger 0,2 cm^3

Attivatori	A O_2 totale consumato dal sistema enzimatico	B O_2 impiegato per la formazione di acido acetacetico
α + chetoglutarato	12,20	8,50
α – chetoglutarato + AsO_4^{---}	0,00	0,00
G. T. P.	9,80	7,10
G. T. P. + AsO_4^{---}	8,30	6,00

c) succinil-CoA + R–COOH ⟶ R-CO CoA + ac. succinico;

2) oppure l'energia per la sintesi degli acil-CoA potrebbe essere fornita al sistema enzimatico dal G. T. P. in maniera diretta, e dall'α-chetoglutarico indirettamente tramite il G. T. P.: infatti dall'ossidazione dell'α-chetoglutarato si forma succinil-CoA [reazione *b*)] e l'energia del legame tioestereo di questo può essere trasferita in quello anidridico del G. T. P. [reazione *a*) decorrente da destra a sinistra].

Il fatto che l'arseniato blocchi l'azione attivatrice dell'α-chetoglutarato[2] può accordarsi con tutte due le ipotesi su riferite: infatti in presenza di arseniato il succinil-CoA che deriva dall'ossidazione dell'α-chetoglutarato subisce un processo di scissione arsenolitica e quindi non può essere utilizzato nè per il trasferimento diretto del CoA all'acido grasso [ipotesi 1)] ne per la sintesi del G. T. P. [ipotesi 2)]. Ora, con le richerche qui riferite l'azione dell'arseniato è stata determinata impiegando, quale attivatore del sistema enzimatico, non più α-chetoglutarico, come si era fatto nelle ricerche precedenti, ma G. T. P.: è evidente che in questo caso l'arseniato, provocando la scissione arsenolitica del succinil-CoA eventualmente formatosi secondo la reazione *a*), avrebbe inibito il potere attivatore del G. T. P. soltanto se questo si fosse svolto tramite il succinil-CoA.

Metodi. L'intensità di azione del sistema enzimatico ossidasico degli acidi grassi è stato determinato mediante il metodo precedentemente descritto [1–3], con il quale si misura sia la quantità di O_2 consumata globalmente dal sistema per ossidare l'acido caprilico (Tabella, colonna A) sia la quantità di O_2 impiegata per la formazione dell'acido acetacetico (Tabella, colonna B). L'arseniato è stato aggiunto in quantità di 40 μM, concentrazione che nelle precedenti ricerche è risultata sufficiente a bloccare completamente l'azione attivatrice dell'α-chetoglutarato.

Risultati e discussione. I risultati ottenuti dimostrano che l'arseniato nel mentre inibisce completamente l'azione attivatrice esplicata dall'α-chetoglutarato sul sistema enzimatico ossidasico dei grassi, invece non modifica in maniera statisticamente significativa l'azione attivatrice del G. T. P. Ora, poichè in presenza di arseniato avviene la scissione arsenolitica del succinil-CoA eventualmente formatosi utilizzando l'energia del legame anidridico del G. T. P., si deve concludere che il potere attivatore di questo non si esplica tramite il succinil-CoA, ma piuttosto che il G. T. P. attiva i sistemi enzimatici fornendo direttamente energia per la sintesi degli acil-CoA.

C. R. Rossi e M. Sacchetto

Istituto di Chimica Biologica, Università di Padova (Italia), 20 ottobre 1958.

Résumé

Dans le foie de cobaye l'arseniate ne modifie pas l'activation provoquée par le G. T. P. sur le système enzymatique des acides gras. De ces résultats on peut conclure que le G. T. P. donne directement l'énergie pour la synthèse des acyl-CoA.

Il succinato ed il fumarato quali regolatori dei sistemi enzimatici ossidasici dei grassi. Nota V

Emmelot e Bos[1] riferiscono numerosi dati sperimentali in appoggio all'ipotesi che nei tessuti animali l'intensità di ossidazione degli acidi grassi sia regolata da un lato dalla concentrazione dell'A.T.P., utilizzato per la sintesi degli acil-CoA, d'altro lato dalla concentrazione dell'A. D. P., utilizzato per i processi di fosforilazione legati all'ossidazione dell'acido grasso stesso. Ora nei sistemi enzimatici ossidasici dei grassi le concentrazioni dell'A. T. P. e dell'A. D. P. possono essere regolate da alcune reazioni concomitanti allo svolgersi del processo ossidativo a carico dell'acido grasso: diminuzione di A. D. P. e corrispettiva sintesi di A. T. P. tramite la fosforilazione ossidativa di un acido del ciclo di Krebs o per azione del sistema fosfocreatina-transfosforilasi, diminuzione di A. T. P. e corrispettiva formazione di A. D. P. per azione dell'esocinasi, dell'atipiasi o di altre fosfatasi aspecifiche. Secondo Emmelot e Bos tali sistemi esercitano la massima attività sull'ossidazione dell'acido grasso quando le reazioni su descritte si svolgono con intensità tale da mantenere continuamente nel sistema enzimatico una opportuna concentrazione di A. T. P. e di A. D. P. Con le ricerche qui riferite si è tentato di convalidare o meno tale ipotesi determinando il comportamento dell'intensità di azione dei sistemi enzimatici ossidasici dei grassi in rapporto al variare della concentrazione dell'A. T. P. e dell'A. D. P. Questo è stato ottenuto variando nel sistema la concentrazione del succinato o del fumarato: tali metaboliti vengono ossidati contemporaneamente all'acido grasso e con intensità variabile in rapporto alla loro concentrazione, quindi varia in maniera corrispondente anche la quantità di A. T. P. sintetizzata e quella di A. D. P. tolta al sistema enzimatico.

Metodi. Il sistema enzimatico ossidasico dei grassi è stato preparato dal fegato di cavia con il metodo di Lehninger[2]. Come attivatore si è impiegato A. T. P. (10 μM). Si sono aggiunte inoltre quantità variabili (da 1 a 100 μM) di succinato o fumarato. Mediante la tecnica manometrica di Warburg, e seguendo le modalità prece-

[1] P. Emmelot e C. J. Bos, Enzymologia *17*, 13 (1954); Biochim. biophys. Acta *16*, 620 (1955); *18*, 281 (1955); *19*, 565 (1956).

[2] A. L. Lehninger, J. biol. Chem. *161*, 437 (1945).

O_2/A.T.P. = μM O_2 consumate per l'ossidazione dell'acido caprilico/μM A.T.P. sintetizzato durante l'ossidazione del succinato o fumarato. Caprilato $0{,}7 \times 10^{-3}$ *M*, Mg^{++} 1×10^{-2} *M*, tampone Na–K–fosfato pH 7,4, 2,4cm³, preparato enzimatico secondo Lehninger 0,2 cm³

μM succinato agglutinato	μM A.T.P. sintetizzato % mg enzima secco	O_2/A.T.P.	μM fumarato agglutinato	μM A.T.P. sintetizzato % mg enzima secco	O_2/A.T.P.
μM 1	55	0,22	μM 1	20	0,20
μM 4	70	0,21	μM 4	35	0,18
μM 10	110	0,19	μM 10	57	0,19
μM 100	152	0,09	μM 100	97	0,04

dentemente descritte[3], si è misurata: *a*) la quantità di O_2 consumata per l'ossidazione del solo succinato o fumarato, e da questa si è risaliti alla quantità di A. T. P. sintetizzato calcolando un P/O di 2 per il succinato e di 3 per il fumarato.

b) la quantità di O_2 consumata per l'ossidazione dell'acido caprilico. I valori ottenuti, esposti nella Tabella, sono riferiti a 100 mg di enzima secco e sono espresso in μM O_2 consumate per l'ossidazione dell'acido caprilico/μM A. T. P. sintetizzato tramite l'ossidazione del succinato o del fumarato (O_2/A. T. P.).

Risultati e discussione. Le ricerche compiute impiegando il succinato dimostrano che il rapporto O_2/A. T. P. (μM O_2 impiegate per ossidare l'acido caprilico/μM A.T.P. sintetizzate tramite l'ossidazione del succinato) diminuisce quando la quantità di A.T.P. sintetizzata supera il valore di 110 μM/100 mg di enzima secco. Per rendere ragione di tale comportamento si potrebbero prospettare queste ipotesi: *a*) alla concentrazione di 110 μM di A.T.P. è completamente saturata la capacità ossidativa del sistema; *b*) la fosforilazione legata all'ossidazione del succinato è limitata dalla disponibilità di A. D. P. e pertanto si svolge solo fino a che la quantità di A. T. P. sintetizzata raggiunge il valore di 110 μM: in tal caso, dato che a questo livello di A. T. P. l'ossidazione del succinato sarebbe dissociata dalla fosforilazione, risulta errata per eccesso la quantità di A. T. P. calcolata in base al rapporto P/O = 2: di qui la diminuzione del rapporto O_2/A. T. P. Riteniamo che tutte due queste ipotesi non possano essere sostenute perché a valori di A. T. P. superiori a 110 μM l'ossidazione dell'acido grasso è diminuita anche in senso assoluto: infatti per 110 μM di A. T. P. sintetizzato il consumo di O_2 per l'ossidazione del caprilato è di 20 μM; e per 150 μM di A. T. P. il consumo di O_2 è di sole 14 μM. Bisognerebbe allora ammettere che l'elevata ossidazione del succinato inibisca l'ossidazione del caprilato interferendo con fenomeni di competizione per l'A. D. P. o per i trasportatori di idrogeno.

Nei riguardi del fumarato viene innanzitutto confermato quanto è stato riferito in altra nota [3], vale a dire il rapporto O_2/A. T. P. non varia fino a quando la quantità di A. T. P. sintetizzata raggiunge il valore di 50 μM. Le ricerche qui riferite precisano che per valori di A. T. P. superiori a 50 μM il rapporto O_2/A. T. P. diminuisce, mentre per il succinato si erano raggiunti valori di 110 μM di A. T. P. Per rendere ragione di tale diversità di comportamento tra il fumarato ed il succinato, si potrebbe prospettare che nel caso del fumarato possano verificarsi fenomeni di competizione fra due sistemi enzimatici ossidasici – quelli interessati nell'ossidazione del fumarato e quelli nell'ossidazione del caprilato – per il D. P. N. Ciò non avviene per il succinato perchè l'ossidazione di questo fino alla tappa che porta alla formazione di fumarato non richiede l'intervento del D. P. N.

M. Sacchetto e C. R. Rossi

Istituto di Chimica Biologica, Università di Padova (Italia), 20 ottobre 1958.

[3] M. Sacchetto e C. R. Rossi, Exper. *14*, 253 (1958).

Résumé

Dans le foie de cobaye, l'activité du système enzymatique oxydasique des acides gras est renforcée par une faible oxydation des acides succiniques et fumariques, tandis qu'elle est inhibée par une oxydation élevée. Il s'agit, peut-être, d'une concurrence pour l'ADP ou bien pour les transporteurs d'hydrogène.

Neue substituierte Indolderivate mit sympathicolytischer und blutdruckaktiver Wirkung

Zu einigen der gebräuchlichsten Hypotonica und Sympathicolytica gehören die Alkaloide der Rauwolfia, der Yohimbe-Rinde und des Mutterkorns. Die aus diesen Drogen isolierten Reinalkaloide haben ihr gemeinsames Merkmal darin, dass sie chemisch zu den Indol-Alkaloiden gehören. Wir haben es deshalb unternommen, einige einfache Indolderivate, die an den C-Atomen 2 und 3 durch verschiedene Reste substituiert sein können, und die am N-Atom durch den Imidazolinylmethylrest alkyliert sind, herzustellen und auf ihre sympathicolytische und blutdruckbeeinflussende Wirkung hin pharmakologisch zu prüfen. In der Tabelle I ist eine Auswahl der dargestellten Verbindungen aufgeführt.

Die sympathicolytische Wirkung dieser Stoffe wurde gegenüber Adrenalin und Arterenol sowohl an der isolierten Rattensamenblase als auch an den peripheren Gefässen der isolierten Kaninchenhinterextremität vergleichend mit Ergotamintartrat und anderen wirksamen Sympathicolytica getestet. Dabei hat sich gezeigt, dass die Indolderivate im Vergleich mit den Indolinderivaten fast ausnahmslos eine bessere sympathicolytische Wirkung entfalten.

Wie aus den Tabellen II und III ersichtlich ist, weist die Substanz XI, das 1-Imidazolinylmethyl-2-β-naphthylindol, sowohl gegenüber Adrenalin als auch gegenüber Arterenol die stärkste antagonistische Wirkung auf.

Die nicht substituierten Indol- resp. Indolinderivate zeigen im Vergleich mit den substituierten Derivaten eine ausgesprochen schwache Sympathicolyse. Ebenfalls von Interesse ist die Tatsache, dass die relativ guten sympathicolytischen Eigenschaften durch Substitution am C_3 abgeschwächt werden. Der Stoff XXIII, das 1-Imidazolinylmethyl-2-phenyl-3-methyl-indol zeigt neben relativ schwacher sympathicolytischer Wirkung an den isolierten Samenblasen einen tonussteigernden Effekt, den wir in der Tabelle mit Eigenwirkung bezeichnet haben. Eine Auswahl der dargestellten Stoffe wurde in bezug auf ihre blutdruckbeeinflussende Wirkung an der mit Numal narkotisierten Katze untersucht. Einige Vertreter, in erster Linie aus der Gruppe der Indolin-Derivate, zeigen starke hypotensive Wirkung. Der Stoff XX, 1-Imidazolinylmethyl-2-p-tolyl-5-chlorindolin weist bei guten

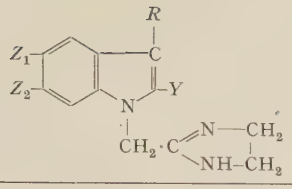
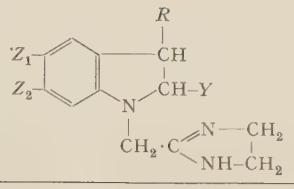


Tabelle I



Indole					Indoline				
Abkürzung	Z_1	Z_2	R	Y	Abkürzung	Z_1	Z_2	R	Y
I	—	—	—	Phenyl-	II	—	—	H	Phenyl-
III	—	—	—	*p*-Tolyl-	IV	—	—	H	*p*-Tolyl-
V	—	—	—	*p*-Äthylphenyl-	VI	—	—	H	*p*-Äthylphenyl-
VII	—	—	—	*p*-Chlorphenyl-	VIII	—	—	H	*p*-Chlorphenyl-
IX	—	—	—	3,4-Xylyl-	X	—	—	H	3,4-Xylyl-
XI	—	—	—	β-Naphtyl-	XII	—	—	H	β-Naphtyl-
XIII	—	—	—	β-Tetralyl-	XIV	—	—	H	β-Tetralyl-
XV	CH_3	—	—	*p*-Tolyl-	XVI	CH_3	—	H	*p*-Tolyl-
XVII	Cl	—	—	*p*-Chlorphenyl	XVIII	Cl	—	H	*p*-Chlorphenyl-
XIX	Cl	—	—	*p*-Tolyl-	XX	Cl	—	H	*p*-Tolyl-
XXI	—	CH_3O	—	*p*-Tolyl-	XXII	—	CH_3O	H	*p*-Tolyl-
XXIII	—	—	CH_3	Phenyl-	XXIV	—	—	CH_3	Phenyl-
XXV	—	—	Phenyl	Phenyl-	XXVI	—	—	Phenyl	Phenyl-

Tabelle II. Sympathicolytische Wirkung an der isolierten Rattensamenblase (Dosierungsverhältnis zur Erzielung gleichen Effektes)

Substanz	Adrenolyse	Arterenolyse	Adrenolyse	Arterenolyse	Substanz
I	9	2,5	2	3,75	II
III	0,3	0,18	0,2	0,19	IV
V	0,1	0,2	0,5	0,21	VI
VII	0,4	0,44	1	0,63	VIII
IX	0,15	0,25	2	0,5	X
XI	0,05	0,1	1	0,38	XII
XIII	0,2	0,38	1	0,5	XIV
XV	0,18	0,25	0,14	0,18	XVI
XVII	1,2	0,63	2	0,95	XVIII
XIX	0,6	1	0,2	0,25	XX
XXI	0,5	0,55	0,4	0,56	XXII
XXIII	1*	1,25*	2	3,75	XXIV
XXV	5	6,25	11	18,75	XXVI
Ergotamintartrat	*1*	*1*	* Eigenwirkung		
Yohimbin	5	5,5			
Phentolamin	0,2	0,25			
Tolazolin	24	22,5			
6-Acetoxy-thymoxyäthyl-dimethylamin	1,2	2,5			

Tabelle III. Sympathicolytische Wirkung am peripheren Gefäss der isolierten Kaninchenhinterextremität (Dosierungsverhältnis zur Erzielung gleichen Effektes)

Substanz	Adrenolyse	Arterenolyse	Adrenolyse	Arterenolyse	Substanz
I	1,48	1,46	3,91	2,0	II
III	0,43	0,26	0,39	0,27	IV
V	0,21	0,22	0,48	0,58	VI
XI	0,17	0,16	0,69	0,35	XII
XIII	0,76	1,15	0,87	0,67	XIV
XIX	1,3	0,65	0,35	0,35	XX
XXI	0,57	0,38	0,65	0,26	XXII
Ergotamintartrat	*1*	*1*			
Yohimbin	1,52	0,54			
Phentolamin	0,43	0,38			
Tolazolin	74	38			
6-Acetoxy-thymoxy-äthyl-dimethylamin	0,69	0,88			

Tabelle IV

Blutdruckwirkung an der Numal-narkotisierten Katze [Effekt in % gegenüber der Ausgangslage nach intravenöser Applikation von 1 mg/kg (Mittelwerte)]

Substanz	nach 60 min	nach 6 h	nach 60 min	nach 6 h	Substanz
III	−12,5	− 7,5	− 21	− 28,5	IV
V	− 7	0	0	+ 14	VI
XI	+ 4	+ 4	+ 8	+ 10	XII
XIII	+ 32,5	+ 21	+ 19,5	+ 10,5	XIV
XVII	+ 15	+ 9	0	− 14	XVIII
XIX	+ 11,5	+ 1,5	− 21	− 20	XX
XXI	− 10,5	0	− 35	− 15	XXII
Reserpin	− 18	− 29			
Phentolamin	− 20,5	− 7,5			
Hydralazin	− 19,5	0			
6-Acetoxy-thymoxy-äthyl-dimethylamin	− 19,5	− 11			

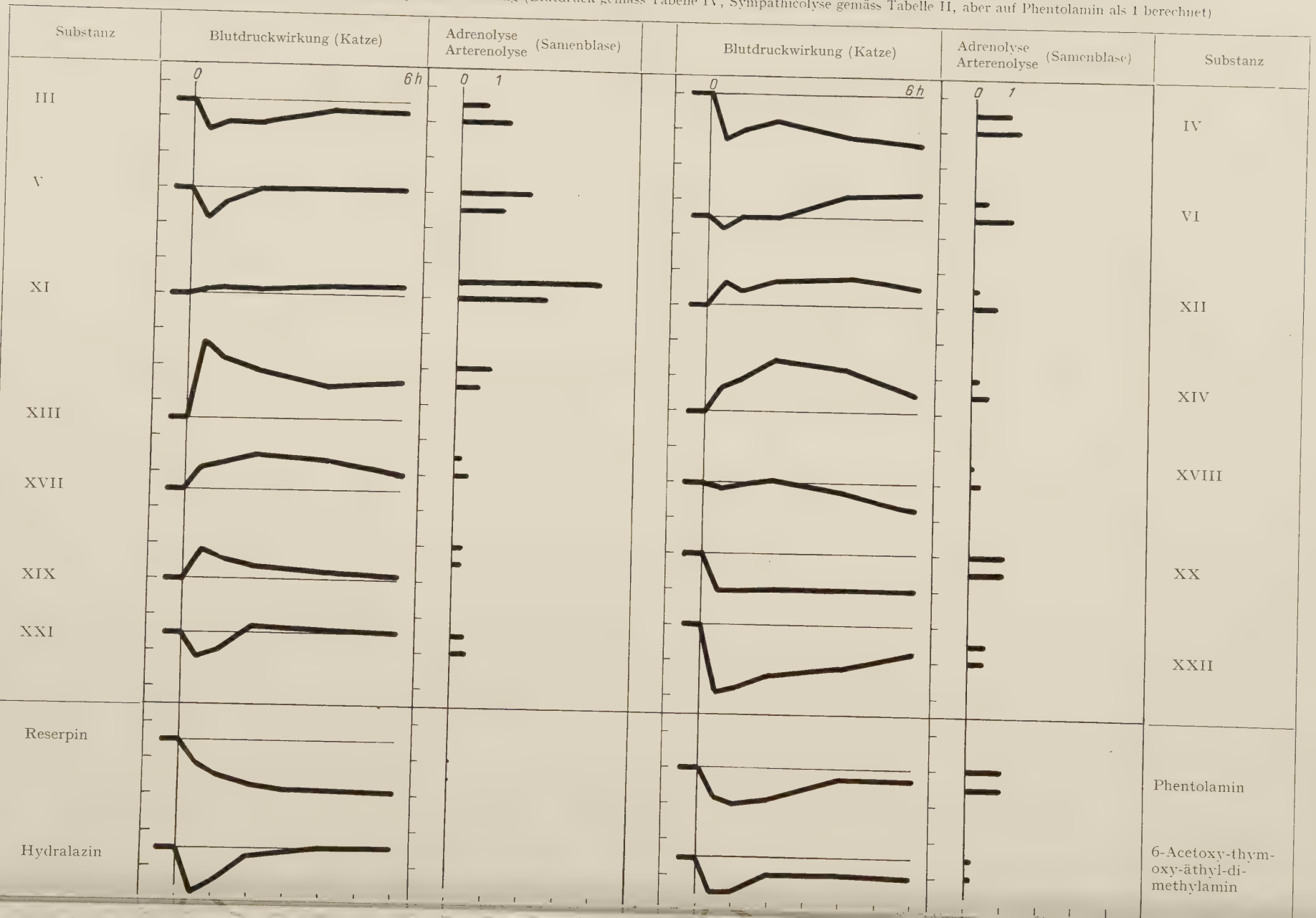
Tabelle VI

Akute Toxizität als DL/50 in mg/kg

Substanz	Ratte		Ratte		Substanz
	intravenös	per os	intravenös	per os	
III	19	240	12,5	250	IV
V	14	310	18	900	VI
XI	19	480	16	1375	XII
XIII	15	730	20	900	XIV
XIX	15	443	32	2150	XX
XXI	29	220	22	595	XXII
Reserpin	25	2000 (Orientierung)			
Phentolamin	82	550			
Tolazolin	96	940			
Hydralazin	56	570			
6-Acetoxy-thymoxy-äthyl-dimethylamin	49	900			
Yohimbin	11	90			
Ergotamintartrat	80	900–1000 (Orientierung)			

Tabelle V

Vergleich von Blutdruck- und sympathicolytischer Wirkung (Blutdruck gemäss Tabelle IV, Sympathicolyse gemäss Tabelle II, aber auf Phentolamin als 1 berechnet)



hypotensiven Eigenschaften eine geringe Toxizität auf, vor allem bei oraler Applikation.

Es scheint uns bemerkenswert, dass unter diesen Stoffen, die sich alle durch eine mehr oder weniger starke sympathicolytische Wirkung auszeichnen, einige Vertreter starke hypertensive Wirkung zeigen, wobei diese vor allem in der Gruppe der Indolderivate anzutreffen sind. Die Substanz XIII, das 1-Imidazolinylmethyl-2-β-tetralylindol zeichnet sich von diesen Substanzen durch stärkste Wirkung aus.

H. L. Küng und W. Schindler

Forschungslaboratorien der J. R. Geigy A.G., Basel, 15. November 1958.

Summary

A series of new substituted indole derivatives was prepared, all of which were distinguished by sympathicolytic activity. A number of these substances, mainly those from the group of indoline derivatives, have a hypotensive action. Others, especially those from the group of indole derivatives, have a hypertensive action.

Über die Kreislaufwirkung des 1-Imidazolinylmethyl-2-β-naphthylindol (G 27032) beim gesunden Menschen

In 48 Versuchen haben wir den adrenolytischen sowie arterenolytischen Effekt des 1-Imidazolinylmethyl-2-β-naphthylindol (G 27032)[1,2] (gegenüber einer konstanten Adrenalin- und Arterenoldosis von 25 γ (Injektionsdauer 15 s) bei gesunden Menschen geprüft. Die wirksame Dosis des Präparates, bei der eine erhebliche Abschwächung oder Aufhebung der Katecholaminkreislaufwirkung eintritt, wurde mit derjenigen von 20 mg Phentolamin[3] verglichen. Die Injektionen erfolgten intravenös bei der liegenden Versuchsperson. Für die Untersuchungen haben sich Kollegen und Studenten freiwillig zur Verfügung gestellt. Injektionsdauer von 1-Imidazolinylmethyl-2-β-naphthylindol und Phentolamin: 90 s. Zur Objektivierung der Kreislaufeffekte registrierten wir den arteriellen Druck mit dem Lilly-Kondensatormanometer (Firma Technitrol Engineering Comp., Philadelphia) und intraarteriellem Katheter. Pulsfrequenzschreibung nach Matthes[4]. An zwei Versuchspersonen wurde die Wirkung von 1-Imidazolinylmethyl-2-β-naphthylindol auf die Haut und Muskeldurchblutung untersucht. Methodik: Kalorimetersonde und Strömungskalorimeter nach Hensel[5].

Zusammenfassung der Ergebnisse

1. Nach Gabe von 20 mg 1-Imidazolinylmethyl-2-β-naphthylindol tritt bei gesunden Menschen eine erhebliche Abschwächung oder Aufhebung der Adrenalin- und Arterenol-Kreislaufwirkung ein (Abb.).

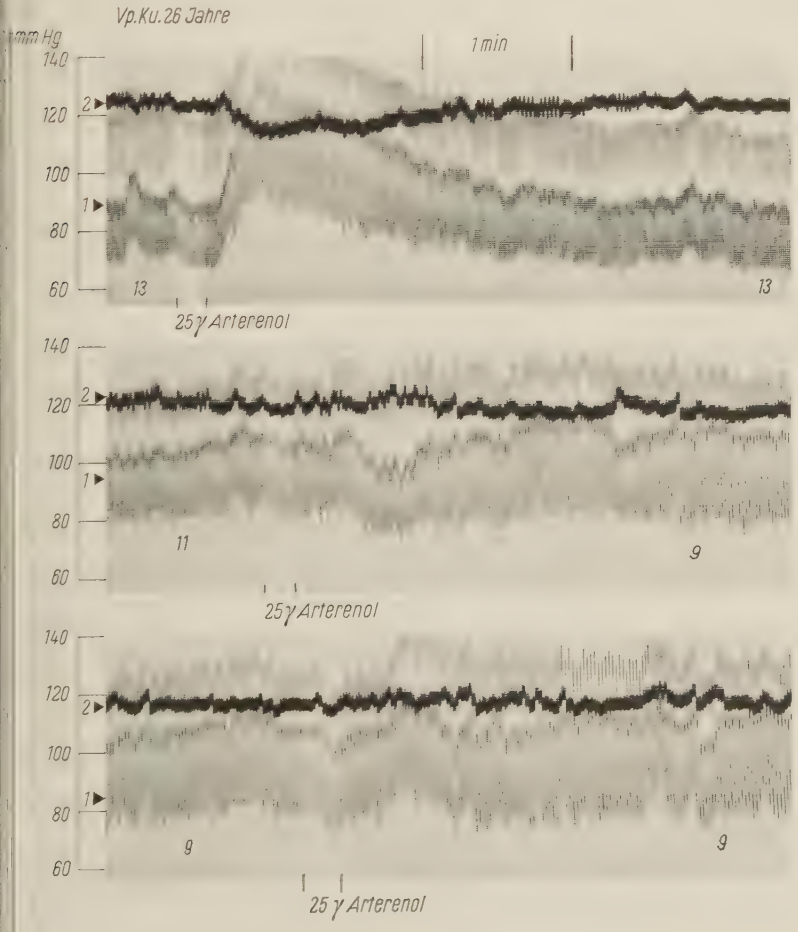
[1] H. L. Küng und W. Schindler, Exper. *15*, 66 (1959).

[2] Diese Substanz wurde uns freundlicherweise von der Firma J. R. Geigy A.G. zur Verfügung gestellt.

[3] Phentolamin = Regitin CIBA.

[4] K. Matthes, *Kreislaufuntersuchungen am Menschen mit fortlaufend registrierenden Methoden* (Stuttgart 1951).

[5] H. Hensel und J. Ruef, Pflügers Arch. *259*, 267 (1954). – H. Hensel, Z. exp. Med. *117*, 587 (1951).



Obere Kurve:

Arterenol-Kreislaufwirkung (25 γ in 15 Sekunden intravenös) vor Injektion von 1-Imidazolinylmethyl-2-β-naphthylindol.

Mittlere Kurve:

Arterenolytischer Effekt. Arterenolwirkung 5 min nach Injektionsende von 20 mg 1-Imidazolinylmethyl-2-β-naphthylindol.

Untere Kurve:

Arterenolwirkung 20 min nach Injektionsende von 20 mg 1-Imidazolinylmethyl-2 β-naphthylindol.

1. Blutdruck — 2. Pulsfrequenz

Die auf der Kurve eingetragenen Zahlen geben die Frequenz für je 10 s wieder.

2. Die gleiche Wirkung kann mit 20 mg Phentolamin erzielt werden (vergleichende Untersuchungen gleicher Dosen von Phentolamin und 1-Imidazolinylmethyl-2-β-naphthylindol bei derselben Versuchsperson: 16 Versuche).

3. Der arterenolytische und adrenolytische Effekt wurde durch wiederholte Injektionen von Adrenalin und Arterenol bei beiden Präparaten für die Dauer von mindestens 20 min nachgewiesen.

4. Im Gegensatz zu Phentolamin beobachtet man nach 1-Imidazolinylmethyl-2-β-naphthylindol bei der Mehrzahl der Personen eine unterschiedlich stark ausgeprägte Abnahme der Herzschlagfolge. Diese Feststellung bezieht sich nur auf einen Vergleich der Pulsfrequenzwerte vor der Injektion von 1-Imidazolinylmethyl-2-β-naphthylindol mit denjenigen vor den nachfolgenden Adrenalin- oder Arterenolinjektionen. Dabei muss berücksichtigt werden, dass in jedem Versuch mit einer Adrenalin- oder Arterenolinjektion begonnen wurde und die Gaben von 1-Imidazolinylmethyl-2-β-naphthylindol erst nach Abklingen des Katecholaminkreislaufeffektes (Blutdruck- und Pulsfrequenz-Normalisierung) erfolgten.

5. Auch die während einer Arterenol- oder Adrenalin-Dauerinfusion auftretenden Blutdruckänderungen werden durch 1-Imidazolinylmethyl-2-β-naphthylindol aufgehoben trotz Fortsetzung der Infusion (Dauerinfusionsdosis 30 γ/min).

K. Mechelke und E. Nusser

Medizinische Universitätsklinik Heidelberg, 15. November 1958.

Summary

In healthy human subjects, 1-imidazolinylmethyl-2-β-naphthylindole, like phentolamine, can greatly weaken or completely abolish the effect of adrenaline or noradrenaline on the circulation. In contrast to phentolamine, however, 1-imidazolinylmethyl-2-β-naphthylindole causes a decrease in heart-rate.

Über den Nukleinsäuregehalt des normalen und hypertrophischen Kaninchenherzens[1]

In der hypertrophischen Herzmuskelfaser ist die Kern-Plasma-Relation zuungunsten des Zellkernes verändert (Linzbach[2]). Obwohl auch die Kerne während der Hypertrophie wachsen und sich amitotisch teilen können, nimmt bei fortschreitender Zunahme der Muskelmasse das Kernvolumen nur in geringerem Masse zu als das Faservolumen. Diese Beobachtung legt die Annahme nahe, dass sich bei der Hypertrophie auch der Gehalt des Myokards an Desoxyribonukleinsäuren ändern wird, da DNS nur im Zellkern, und zwar in den Chromosomen vorkommt.

Diese Frage wurde an Kaninchenherzen untersucht, bei denen durch künstliche Aorteninsuffizienz eine Hypertrophie des linken Ventrikels verursacht wurde. Die Aorteninsuffizienz wurde durch Zerstörung einer Semilunarklappe von der rechten Arteria carotis aus unter Druckkontrolle erzeugt. Die linken Ventrikel von 9 operierten Tieren wurden 6 Monate nach dem Eingriff auf ihren Gehalt an Ribonukleinsäuren (RNS-P) und Desoxyribonukleinsäuren (DNS-P) mit der Methode von Schmidt und Thannhauser[3] untersucht. Die Phosphorbestimmungen wurden mit der Methode von Fiske und Subbarow[4] ausgeführt. 12 normale Tiere dienten zum Vergleich. Es wurden nur männliche Tiere verwendet. Das Ausmass der durch die Operation hervorgerufenen Hypertrophie geht aus Tabelle I hervor. Herzgewicht, Ventrikelgewicht und der Index Herzgewicht/Tiergewicht sind bei den Tieren mit Aorteninsuffizienz deutlich erhöht. Die Blutdruckamplitude der operierten Tiere ist im Durchschnitt um fast 150% erhöht.

Tabelle I

	Normal	Hypertrophie
Tiergewicht (kg)	3,4	3,8
Herzgewicht (g)	8,6	13,0
Ventrikelgewicht (g) (links + rechts)	7,0	11,0
Herzgewicht (g) / Tiergewicht (kg)	2,5	3,4
Blutdruckamplitude (Carotis) mm Hg	20	49

Tabelle II

Mittelwerte und mittlere Fehler der Nukleinsäure-Anteile von 12 normalen und 9 hypertrophischen linken Ventrikeln.
mg P/100 g Frischgewicht

	Normal	Hypertrophie
Gesamt-P	36,41 ± 1,0299	35,93 ± 1,7672
Anorg.-P	1,95 ± 0,2414	1,77 ± 0,2159
DNS-P	8,71 ± 0,2916	6,81 ± 0,2963
RNS-P	25,76 ± 0,8785	27,36 ± 1,4294

In Tabelle II sind die Ergebnisse der Nukleinsäurebestimmungen zusammengefasst. Gesamtphosphor bedeutet Nukleinsäure-P + Phosphorprotein-P. Anorganischer P bedeutet Phosphoprotein-P.

Beim Gesamt-P und anorganischen P liegen keine statistisch wesentlichen Unterschiede zwischen den beiden Tiergruppen vor. Bei den DNS und RNS unterscheiden sich die Mittelwerte der Normaltiere wesentlich von den hypertrophischen Herzen (Irrtumswahrscheinlichkeit 0,1%). Das Verhältnis DNS-P/RNS-P verschiebt sich von 1:2,9 bei den Kontrollen auf 1:4 bei den hypertrophischen Herzen.

Aus den Ergebnissen geht hervor, dass bei einer Hypertrophie mittleren Grades der Gehalt des Myokards an Desoxyribonukleinsäuren gegenüber normalem Myokard vermindert ist, während der Ribonukleinsäuregehalt erhöht erscheint. Das Verhalten der DNS entspricht den Erwartungen, da der DNS-Gehalt der Zelle sich normalerweise nur durch Mitose vermehrt, während die Massenzunahme bei der Herzhypertrophie nur von der Grössenzunahme des Kernes und amitotischen Kernteilungen begleitet ist. Es scheint so, als würde das Verhältnis der nur im Zellkern lokalisierten DNS zur RNS, die in Kern und Zytoplasma gefunden wird, die Veränderungen der Kern-Plasma-Relation wiedergeben.

Einzelheiten der Methodik, Einzeldaten und weitere Versuchsergebnisse werden an anderer Stelle ausführlich mitgeteilt.

H. Nowy, H. D. Frings und K. Rey[5]

Medizinische Poliklinik der Universität München, 15. Oktober 1958.

[1] Mit Unterstützung der Deutschen Forschungsgemeinschaft.

[2] A. J. Linzbach, Handbuch der allgemeinen Pathologie, Bd. VI/1 (Springer-Verlag, Berlin-Göttingen-Heidelberg 1955), p. 180.

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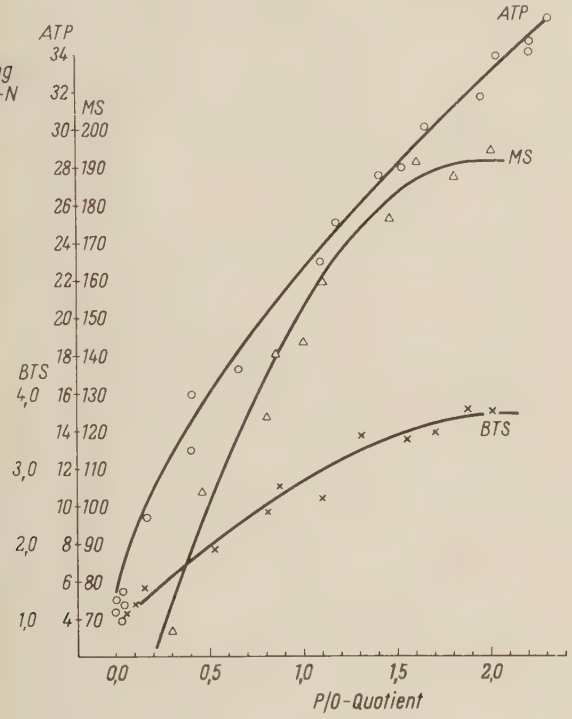
Summary

In left ventricular myocardium of 9 hypertrophic (artificial aortic insufficiency) and 12 normal rabbit hearts, ribonucleic and desoxyribonucleic acids were determined. In hypertrophy DNA-P was significantly reduced, whereas RNA-P was higher than in normal myocardium. The RNA-P/DNA-P ratio was 2·9 in normal as compared to 4·0 in hypertrophic heart muscle.

Die oxydative Phosphorylierung isolierter Mitochondrien und ihre Abhängigkeit von strukturgebundenen Faktoren

Untersuchungen von Raaflaub[1] *et al.* haben gezeigt, dass isolierte Mitochondrien zu schwellen beginnen, wenn der intramitochondriale Gehalt an Adenosintriphosphat (ATP) unter einen kritischen Wert sinkt. Im Warburg-Versuch bewirkte die Abnahme der strukturgebundenen ATP eine Hemmung der Oxydationsfähigkeit der Mitochondrien für Substrate des Zitronensäurezyklus: die Sauerstoffaufnahme ging kontinuierlich zurück.



Abhängigkeit der Höhe des P/O-Quotienten vom Gehalt an intramitochondrialer ATP, MS und BTS

In Ergänzung dieser Untersuchungen interessierte uns, ob auch für die Phosphorylierungsfunktion der Mitochondrien ähnliche Abhängigkeiten von strukturgebundenen Faktoren nachgewiesen werden können.

Methodik. In zwei Versuchsreihen (90 Einzelversuche) wurde der P/O-Quotient intakter und durch «ageing» geschwollener Mitochondrien aus Kalbs- und Rinderlebern mit Ketoglutarat/Malonat bzw. Succinat als Substrat ermittelt. Bei jedem Einzelversuch wurden die Mitochondrien gleichzeitig auf ihre Konzentration an strukturgebundenen Adenosinnukleotiden, an intramitochondrialer Milchsäure (MS), Brenztraubensäure (BTS) und α-Ketoglutarsäure (KGS) mittels optisch-enzymatischer Methoden untersucht; ausserdem wurde ihr Gehalt an strukturgebundenem Eisen und Kupfer auf chemischem Wege bestimmt.

Ergebnisse. Die Fähigkeit isolierter Mitochondrien zur Durchführung der oxydativen Phosphorylierung ist von einem bestimmten Gehalt an intramitochondrialen Adenosinphosphaten, Substraten und Schwermetallen abhängig. Abnahme dieser Faktoren bewirkt eine Senkung des P/O-Quotienten, wobei die Phosphorylierungsfunktion wesentlich schneller geschädigt wird als die Oxydationsfähigkeit.

Die optimale Konzentration – gemessen an der Höhe des P/O-Quotienten – betrug für Adenosinmonophosphat 0,009 μM, Adenosindiphosphat 0,017 μM und ATP 0,063 μM/mg Mitochondrien-Stickstoff.

Für KGS lagen die optimalen Werte bei 3,00 γ, für BTS bei 3,75 γ, für MS bei 195,0 γ, für Fe bei 1,29 γ und für Cu bei 2,56 γ/mg Mitochondrien-Stickstoff.

Besonders der Gehalt an intramitochondrialer ATP, BTS und MS steht in einer echten Korrelation zur Funktion des phosphatübertragenden Systems: eine Abnahme der genannten Konzentrationen senkt kontinuierlich die Phosphataufnahme und erniedrigt dadurch den P/O-Quotienten (vgl. Abb.); die Oxydationsfähigkeit bleibt zunächst voll erhalten. Erst wenn eine Grenzkonzentration von etwa 0,01 μM gebundener ATP und BTS sowie etwa 0,4 μM/mg-N MS unterschritten wird, wird auch die Oxydationsfähigkeit der Mitochondrien für exogenes Substrat zunehmend gehemmt. Zu diesem Zeitpunkt setzen mit der Schwellung die strukturellen Schäden ein.

Eine ausführliche Darstellung der Untersuchungen erfolgt an anderer Stelle.

G. Laudahn

Innere Abteilung des Städtischen Krankenhauses, Berlin-Wilmersdorf, 5. September 1958.

Summary

The ability of isolated mitochondria in coupling phosphorylation to oxidation depends on sufficient concentrations of intramitochondrially bound adenine nucleotides (especially ATP), compounds of oxidative metabolism (pyruvic and lactic acid) and iron ions. Continuous loss of these components by ageing causes at first a parallel lowering of phosphate uptake and does not affect respiration. Later on, a decrease of oxidative ability begins when the loss of intramitochondrial components reaches a limiting concentration, and now mitochondria show more and more swelling and structural damages.

Porter-Silber-Reaktion mit C_{16} substituierten Corticosteroiden

Zur Untersuchung der Nebennierenrindenfunktion des Menschen werden die 17-Hydroxycorticoide im Blute und Urin mit der Reaktion nach Porter-Silber bestimmt[1,2]. Die Methode hat den Vorteil, relativ einfach und spezi-

[1] J. Raaflaub, Helv. phys. Acta *11*, 142, 157 (1953). – O. Brenner-Holzach und J. Raaflaub, Helv. phys. Acta *12*, 242 (1954). – G. Laudahn und E. Rasch, Biochem. Z. *330*, 509 (1958). – G. Laudahn, Z. physiol. Chem. (im Druck).

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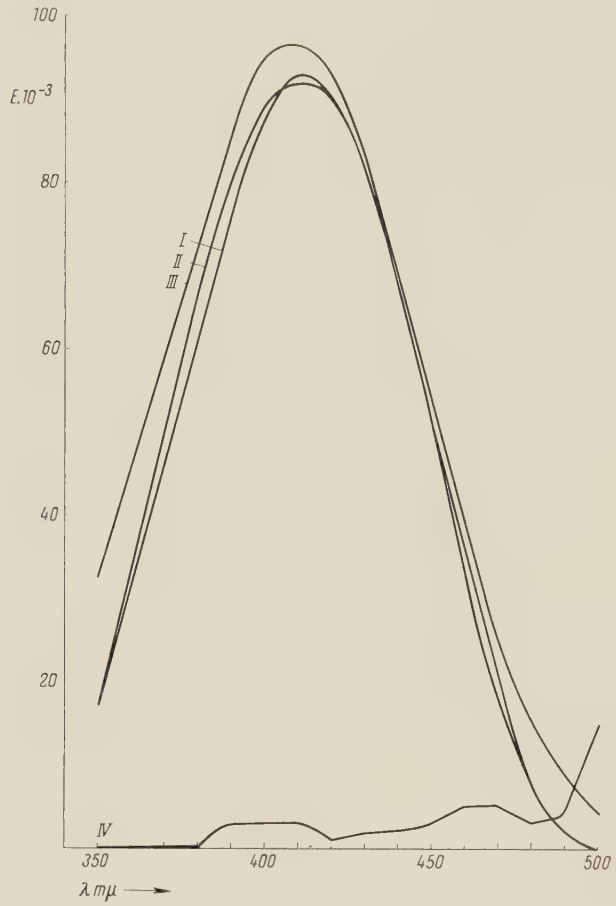
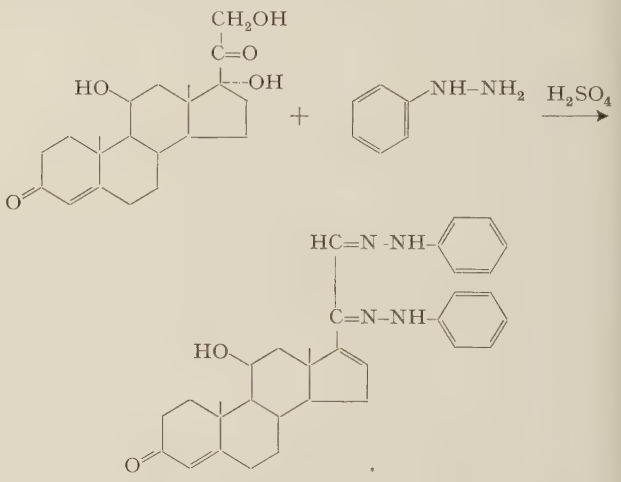
fisch zu sein. Die Spezifität beruht auf der Dihydroxyacetonkonfiguration an C_{17} von Cortisol und den direkten Cortisolmetaboliten, welche mit Phenylhydrazin in Schwefelsäure reagieren[3]. Verschiedene synthetische Corticosteroide, die für therapeutische Zwecke oft verwendet werden, besitzen die gleiche C_{17}-Seitenkette. In letzter Zeit



wurden 2 neue Corticosteroide entwickelt, welche ebenfalls die gleiche Konfiguration an C_{17} besitzen, dazu aber noch an C_{16} substituiert sind. Triamcinolon ist 9α-Fluoro-16α-Hydroxyprednisolon und Hexadecadrol ist 9α-Fluoro-16α-Methylprednisolon. Beide Stoffe sind pharmakologisch stark wirksam und führen zu einer Hemmung der unter der Kontrolle von ACTH stehenden Nebennierensteroide. Es wurde deshalb untersucht, ob diese C_{16} substituierten Steroide zu einer Veränderung der Porter-Silber-Reaktion und ihrem typischen Absorptionsmaximum bei 410 mμ führen. Die Steroide wurden aus der Handelsform durch Extraktion mit Aceton erhalten. Die Porter-Silber-Reaktion wurde in üblicher Weise mit Lösung von 10–100 γ Substanz in Methanol mit Phenylhydrazin-Schwefelsäure während 20 min bei 60°C ausgeführt. Nach Abkühlen wurde mit Quarzküvetten im Zeiss-Spektralphotometer PMQ II photometriert. Als Vergleich dienten aliquote Lösungen von Cortisol und 9α-Fluorocortisol in Methanol.

Die Bestimmung der Absorptionskurve der 4 Lösungen (je 10 γ) ergab für Cortisol (Kurve I), 9α-Fluorocortisol (Kurve II) und 9α-Fluoro-16-Methylprednisolon (Hexadecadrol) (Kurve III) identische Werte mit Maximum bei 410 mμ. 9α-Fluoro-16-Hydroxyprednisolon (Triamcinolon) (Kurve IV) ergab keine Porter-Silber-Absorptionskurve. Die Ergebnisse der Versuche mit höheren Konzentrationen waren entsprechend. Diese Resultate weisen darauf hin, dass die bisherige Annahme der Bildung eines Osazones aus Phenylhydrazin und der Dihydroxyacetonkonfiguration von Cortisol, resp. Hexadecadrol, unter den gegebenen Versuchsbedingungen nicht genügen kann, weil Triamcinolon keine typische Porter-Silber Reaktion gibt. Man muss vielmehr annehmen, dass ausser der Osazonbildung noch zwischen C_{16} und C_{17} eine Wasserabspaltung stattfindet. Die dabei entstehende Doppelbildung zwischen C_{16} und C_{17} muss für die spezifische Absorption von Bedeutung sein. Die positive Reaktion von Hexadecadrol schliesst die Möglichkeit aus, dass eine Wasserabspaltung bei Triamcinolon durch eine sterische Hinderung nicht stattfinden könnte. Wir vermuten, dass beim Triamcinolon durch Wasserabspaltung ein Enol entsteht, das nicht stabil ist und in das entsprechende Keton übergeht. Im gleichen Sinne sprechen die Versuche von Mattox *et al.*[4]. Diese Autoren stellten fest, dass Δ-16-Pregnen-3α-21-diol, 11, 20-dion, 3, 21-diacetat in typischer Weise mit Phenylhydrazin reagiert. Es ist in diesem Zusammenhang von Interesse, dass ein neues 21-Desoxycorticosteroid durch die Porter-Silber-Reaktion nicht bestimmt werden kann[5].

Bei der Durchführung klinischer Versuche mit Triamcinolon dürfen die 17-Hydroxycorticoide deshalb nicht mit der Porter-Silber-Reaktion bestimmt werden. Wir nehmen an, dass das Reaktionsendprodukt der Porter-Silber-Reaktion zwischen Phenylhydrazin und Cortisol folgende Struktur aufweist:



A. Walser und H. P. Schlunke

II. Med. Abteilung, Bürgerspital Basel, 18. November 1958.

Summary

The determination of 17-hydroxycorticoids by the Porter-Silber reaction depends on a typical absorption curve with maximum at 410 mμ. Hexadecadrol with 16-methyl group gives this typical curve but not Triamcinolon containing a hydroxylgroup at C_{16}. It is assumed that besides the formation of an osazone a double bound between C_{16} and C_{17} occurs, which is essential for a typical Porter-Silber reaction. This has to be considered in the determination of 17-hydroxycorticoids of patients treated with C_{16} corticosteroids.

[3] E. Diczfalusy, G. Birke und L.-O. Plantin, Klin. Wschr. *34*, 225 (1956).

[4] V. R. Mattox, H. L. Mason und A. Albert, Proc. Staff Meetings Mayo Clin. *28*, 569 (1953).

[5] M. B. Lipsett und D. M. Bergenstal, J. clin. Endocrinol. Metab. *18*, 790 (1958).

Influence of Iproniazid Pretreatment on Formation of Gastric Ulcers by Reserpine in the Rat

Reserpine, besides its effects on the central nervous and cardiovascular system, has an important influence on the gastro-intestinal tract. This drug stimulates gastric secretion and motility[1–6] and increases the production of gastric hemorrhagic erosions after ligation of the pylorus in rats[7,8]. It has been demonstrated that this substance releases serotonin from the brain and intestines[9,10]. The central nervous effects of reserpine are markedly changed after pretreatment with iproniazid, an inhibitor of monoamine oxidase, which prevents the decreases of serotonin in the brain by reserpine[11,12]. This suggests that the central pharmacological actions of reserpine might be due at least partly to alterations in the monoamine metabolism in the brain[13].

The study of the influence of pretreatment with iproniazid (Marsilid®) on the gastro-intestinal effects of reserpine might give additional informations on the working mechanism of reserpine.

In a first series of experiments, the possible inhibitory influence of iproniazid-pretreatment on the production of gastric erosions in the Shay-rat preparation was examined. 60 rats were divided in 3 groups. One group served as a control, a second group received 5 mg/kg reserpine intraperitoneally at the time of the ligation of the pylorus, and the third group was injected with iproniazid (100 mg/kg intraperitoneally 5 h before ligation of the pylorus) and reserpine (5 mg/kg at the time of pyloric ligation). Ligation of the pylorus was performed under light ether anesthesia. At least 48 h prior to operation all food had been removed. Water was withheld from the time of operation until sacrifice. $7^1/_2$ h after the pylorus ligation, the rats were killed, their stomach removed, the accumulated gastric juice collected, and the gastric mucosa examined for ulcers or erosions.

The results show that iproniazid markedly inhibits the production of gastric hemorrhagic erosions by reserpine. Indeed, while all the animals which had received reserpine alone, showed hemorrhagic erosions of the glandular part of the stomach [antrum and (or) corpus] $7^1/_2$ h after pyloric ligation, no such lesions were observed in the reserpine animals which had been pretreated with iproniazid. The rats of the control group also did not show gastric erosions after the same time interval.

The mean values of the pH, free acidity, and total volume of the collected gastric juice showed no significant difference between the reserpine group and the iproniazid-reserpine groups.

It has been found that the serotonin precursor 5-hydroxytryptophan inhibits gastric acid secretion[14]. Yet, consistently lesions of the glandular portion of the gastric mucosa were found in rats after a single injection of a high amount of this substance[15]. Reserpine on the other hand, which decreases and liberates the serotonin in the gastro-intestinal tract, increases gastric acid secretion and ulcer formation. Iproniazid, which is known to interfere with the metabolism of the monoamines inhibits the production of gastric erosions by reserpine. The possibility exists that this protective effect depends on the interference of this substance with serotonin metabolism.

I. Leusen, E. Lacroix, and G. Demeester

Laboratory of Physio-Pathology of the University of Ghent, October 9, 1958.

Résumé

Chez le rat à pylore lié, la réserpine (5 mg/kg) provoque l'apparition précoce d'ulcérations gastriques. Ce phénomène fait défaut si les rats ont été traités préalablement par l'iproniazide, un inhibiteur de la monoamino-oxydase.

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[4] E. W. Barrett, R. A. Rutledge, and B. Rogie, Fed. Proc. *13*, 384 (1954).

[5] W. E. Barret, A. J. Plummer, E. A. Earl, and B. Rogie, J. Pharmacol. *113*, 3 (1955).

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[15] B. J. Haverback and D. F. Bogdanski, Proc. Soc. exp. Biol. Med., N. Y. *95*, 392 (1957).

Kompetitiver Antagonismus zwischen g-Strophanthin und Corticosteron an isolierten Streifen von Rattenaorten*

Über eine antagonistische Wirkung von DOC-Glycosid gegenüber Azetylcholin am Froschherzen berichtete Gross[1]. Haefeli[2] wies am Froschrectus eine Hemmung der Acetylcholin-Veratrin- und Guanidinkontraktur durch verschiedene Nebennierensteroide, Progesteron und Androgene nach. Wir fanden, dass DOC, Corticosteron und Testosteron die durch Serotonin ausgelöste Kontraktion der Rattenaorta hemmen. Alle diese Effekte sind insofern unspezifischer Natur, als offenbar ganz beliebige Reizstoffe (bzw. Hemmstoffe) durch Steroidhormone antagonisiert werden. Kürzlich zeigte Leonard[3], dass DOC und Progesteron sogar die elektrisch ausgelöste Kontraktion von Kaninchenkarotiden hemmen.

g-Strophanthin erzeugt in hoher Konzentration (10^{-4} g/ml) an Aortenstreifen von Ratten einen langsamen Tonusanstieg. Wir fanden, dass dieser Tonusanstieg durch Corticosteron gehemmt wird. Es stellte sich die Frage, ob es sich auch hierbei um einen unspezifischen Effekt handelt oder ob, wie sich aus der chemischen Verwandtschaft der Substanzen vermuten lässt, ein echter Antagonismus vorliegt. Zur Klärung dieser Frage wurde versucht, die Kinetik der Hemmung zu analysieren.

Thoraxaorten von Ratten wurden etwa 15 h bei 4°C aufbewahrt, in spiralige Streifen (Furchgott und Bhadrakom[4]) von 1–1,5 mm Breite geschnitten und in einer

* Diese Arbeit wurde ermöglicht durch ein Stipendium der «Stiftung der CIBA für naturwissenschaftliche, medizinische und technische Forschung».

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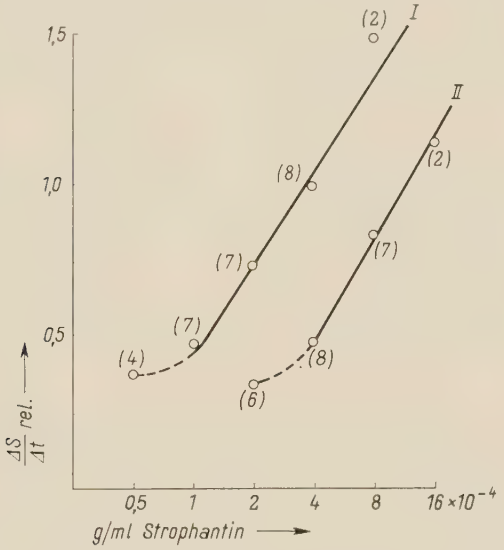
modifizierten Krebslösung mit verringertem Na- und Ca-Gehalt bei 37°C untersucht. Die entwickelte Spannung wurde an einem isometrischen Hebel über eine pneumatische Druckverstärkervorrichtung registriert. Der durch Strophanthin ausgelöste Tonus ist gekennzeichnet durch einen langsamen, annähernd linearen Anstieg (die lineare Phase beginnt nach einer gewissen Latenzzeit oder sogleich nach Zusatz des Strophanthins). Als Mass für die Wirkung der Substanzen wurde die *Geschwindigkeit* dieses Anstiegs verwendet, worauf weiter unten eingegangen wird. Im allgemeinen steigerte sich die Empfindlichkeit des Präparates während der Versuchsdauer, in einigen Fällen dagegen sank sie etwas ab. Es wurden 8 Versuche ausgewählt, bei denen die Empfindlichkeitsänderung in der einen oder anderen Richtung gering war und sich im Mittel aller Versuche aufhob. Die Abbildung gibt das



Abszisse: Strophanthinkonzentration (g/ml) (logarithmisch). Ordinate: $\Delta S/\Delta t$: Änderung der mechanischen Spannung pro Zeiteinheit in relativem Maßstab (Wert bei $4 \cdot 10^{-4}$ Strophanthin ohne Corticosteron in jedem Einzelversuch = 1 gesetzt). I ohne und II mit Corticosteron $5 \cdot 10^{-6}$ (g/ml) (Corticosteron in Alkohol gelöst. Alkohol-Endkonzentration im Bad 0,1%). – Die Zahlen in Klammern geben Anzahl der Versuche pro Punkt, wobei jeder Streifen gewöhnlich für jede Kurve 3 oder 4 verschiedene Messpunkte lieferte.

Resultat der Versuche in der Form der Dosis-Wirkungskurven wieder. Unter Ausschluss der untersten Punkte beider Kurven wurden die Regressionsgeraden (ausgezogen) berechnet. Sie zeigen eine ganz geringe Konvergenz nach oben und sind mit guter Annäherung als parallel zu betrachten. Die Parallelität, das heisst die Gleichheit des horizontalen Abstandes der Dosis-Wirkungskurven über den ganzen Bereich in der halblogarithmischen Darstellung bedeutet, dass für einen gegebenen Effekt das Verhältnis der dafür nötigen Strophanthinkonzentration mit und ohne Corticosteron konstant ist. Dieses Verhalten ist charakteristisch für einen Verdrängungsantagonismus, bei dem der eine Stoff (Strophanthin) (S) an einem Rezeptor (R) der Zelle einen Effekt auslöst, der bestimmt ist durch die Konzentration des Komplexes (RS), während der andere (Corticosteron) (C) am *gleichen* Rezeptor gebunden wird, ohne eine sichtbare Wirkung zu haben. Für einen solchen Mechanismus lässt sich leicht das Folgende ableiten (s. zum Beispiel Ariens *et al.*[5]):

$$(RS) = \frac{(r)}{\frac{K_S(C)}{K_C(S)} + \frac{K_S}{(S)} + 1} \qquad (1)$$

[5] E. J. Ariens, J. M. van Rossum und A. M. Simonis, Arzneimittel-Forsch. *6*, 282 (1956).

wobei

(RS) Konzentration des Komplex Rezeptor-Strophanthin;
(r) gesamte Konzentration des Rezeptors;
(S) Strophanthinkonzentration;
(C) Corticosteronkonzentration;
K_S Dissoziationskonstante des Komplex Rezeptor-Strophanthin;
K_C Dissoziationskonstante des Komplex Rezeptor-Corticosteron.

Umformung von (1) ergibt

$$\frac{(r)}{(RS)} = \frac{K_C(C)}{K_S(S)} + \frac{K_S}{(S)} + 1 . \qquad (2)$$

Wenn $C = 0$, wird (1) zu

$$\frac{(r)}{(RS)_{C=0}} = \frac{K_S}{(S)_{C=0}} + 1 . \qquad (3)$$

Für gleichen Effekt mit und ohne Corticosteron muss $(RS) = (RS)_{C=0}$ sein, so dass:

$$\frac{K_S}{(S)_{C=0}} + 1 = \frac{K_S(C)}{K_C(S)} + \frac{K_S}{(S)} + 1$$

oder

$$\frac{(S)}{(S)_{C=0}} = \frac{(C)}{K_C} + 1 , \qquad (4)$$

das heisst $(S)/(S)_{C=0}$ ist nur abhängig von Konzentration und Eigenschaften von C, nicht aber von der absoluten Grösse von (S), also auch nicht von der absoluten Grösse des Effekts. (4) gilt ganz allgemein, solange gleiche Wirkungen mit und ohne Corticosteron verglichen werden, das heisst $S/S_{C=0}$ ist auch unabhängig vom funktionellen Zusammenhang zwischen Wirkung und Konzentration des Komplex (RS). Das Verhältnis $(S)/(S)_{C=0}$ variiert nicht mit (S), also ist auch die Differenz $\log(S) - \log(S)_{C=0}$ konstant für alle Werte von (S). Der experimentelle Befund paralleler Dosiswirkungskurven ist demnach in Einklang mit der Theorie der kompetitiven Hemmung.

Die Frage, an welchem Mechanismus des Kontraktionsvorganges diese Kompetition stattfindet, ist unbeantwortet. Sulser und Wilbrandt[6] zeigten an Menschenerythrocyten, dass der durch Herzglykoside gebremste aktive Transport von Natrium durch verschiedene Nebennierensteroide restituiert werden kann. Es liegt deshalb nahe zu vermuten, dass auch der hier beobachtete Antagonismus sich auf den Ionentransport durch die Zellmembran beziehen könnte, in dem Sinn, dass Strophanthin ein normalerweise den Kationentransport vermittelndes, chelatbildendes (Wilbrandt[7]) Membransteroid an seiner Haftstelle verdrängt, was zur Änderung der Kationenkonzentration im Zellinneren und damit des Tonus führt, und dass diese Membransteroide durch zugesetzte Nebennierensteroide vertreten werden können. Die in den vorliegenden Versuchen gemessene Geschwindigkeit des Tonusanstiegs könnte unter dieser Annahme Ausdruck der Geschwindigkeit sein, mit der sich die intrazelluläre Ionenkonzentration ändert.

H. J. Schatzmann

Pharmakologisches Institut der Universität Bern, 3. September 1958.

Summary

Corticosterone was shown to have an inhibitory effect on the contraction caused by a cardiac glycoside (g-Strophanthin = Ouabain) in isolated strips of rat aorta. It could be demonstrated that over a tenfold concentration range of Ouabain, the kinetics of this antagonism are in accord with the assumption of a competitive inhibition.

[6] F. Sulser und W. Wilbrandt, Helv. physiol. Acta *15*, C 37 (1957).

[7] W. Wilbrandt, Helv. physiol. Acta *16*, 31 (1958).

The Content of Noradrenaline and Adrenaline in the Rat Heart After Administration of Rauwolfia Alkaloids[1]

Reserpine has been shown to lower the catechol amine content of brain, peripheral sympathetic tissues, adrenal medulla, heart, and artery walls[2–10]. The heart rate increase produced by reserpine in the dog heart-lung preparation has been ascribed by PAASONEN and KRAYER[9] to the noradrenaline released from the heart tissue itself. In connection with the experiments described in the last mentioned publication the effect of a number of Rauwolfia alkaloids on the adrenaline and noradrenaline content of the rat heart was determined.

The assays of the noradrenaline and adrenaline content were made in the heart taken from intact rats. Two hearts were combined for each analysis. The tissues were ground in 5% trichloroacetic acid and the procedure was that described by v. EULER[11], except for minor changes[9]. The samples were analyzed biologically by using the cat blood pressure and the isolated rectal caecum of the hen. Before starting the blood pressure assay a combination of drugs, as used by KÄRKI[12], was administered. The methods and materials have been described by PAASONEN and KRAYER[9].

The Rauwolfia alkaloids (generously supplied by S. B. Penick & Co., N.Y.) were dissolved in glacial acetic acid and the solution diluted so that the concentration of acetic acid injected was not in excess of 2%. The drugs were injected intraperitoneally. The doses of L-noradrenaline monohydrate and L-adrenaline bitartrate (Sterling-Winthrop) are given in terms of the base.

Results. The reserpine-type alkaloids[13], raunescine, isoraunescine, deserpidine (canescine, recanescine), and rescinnamine, upon intraperitoneal injection, produced a marked depletion of noradrenaline in the rat heart at the dose level of 5 mg/kg. The data are assembled in the Table. The intensity of the effect is similar to that observed with reserpine at the same dose level[9]. Even at a level of 20 mg/kg, yohimbine, which is chemically different from the reserpine-type group, had no influence upon the noradrenaline content of the heart.

For a quantitative comparison of the ability of different reserpine-type alkaloids to release noradrenaline, the dose of 5 mg/kg appears to be too high. A clear-cut difference in potency between raunescine and isoraunescine was evident at the dose of 0·5 mg/kg. At this dose, isoraunescine removed only about 50% of the noradrenaline within 4 h, while raunescine was fully as active as at the ten times higher dose. In four experiments, not included in the Table, 0·5 mg/kg of reserpine, within 2 h, removed 69% (range 58–77) of the noradrenaline content of the heart.

Effect of Rauwolfia Alkaloids on the Noradrenaline and Adrenaline Content of the Rat Heart

Substance	Dose (mg/kg)	Time[a] (h)	Individual Values mμg/g[b]	Mean mμg/g[c]
			Noradrenaline	
Control . . .			($n = 13$)	490 ± 140[d]
Isoraunescine	0·5	6	245,250	248
Isoraunescine	5·0	4	68	68
Isoraunescine	5·0	6	28	28
Isoraunescine	5·0	16	<20[e],13	<17
Raunescine .	0·5	6	32,36	34
Raunescine .	5·0	4	34,15	25
Raunescine .	5·0	6	<28[e]	<28
Raunescine .	5·0	16	15,28	22
Deserpidine[f] .	5·0	4	71,30	51
Rescinnamine	5·0	4	17	17
Rescinnamine	5·0	6	<29[e]	<29
Yohimbine .	5·0	6	350,495	423
Yohimbine .	20·0	4	355,550	453
			Adrenaline	
Controls . .			($n = 10$)	32 ± 14[d]
Isoraunescine	0·5	6	42,36	39
Isoraunescine	5·0	4	45	45
Isoraunescine	5·0	6	14	14
Isoraunescine	5·0	16	6,20	13
Raunescine .	0·5	6	26,39	33
Raunescine .	5·0	4	30,27	29
Raunescine .	5·0	6	14	14
Raunescine .	5·0	16	24,24	24
Deserpidine[f] .	5·0	4	28,30	29
Rescinnamine	5·0	4	45	45
Rescinnamine	5·0	6	15	15
Yohimbine .	5·0	6	34,41	38

[a] Time after intraperitoneal injections.
[b] Each individual value is obtained from two pooled hearts.
[c] Wet weight.
[d] ± standard deviation.
[e] (<) Not detectable and less than the value presented.
[f] Also called canescine or recanescine.

Adrenaline amounted to about 6% of the noradrenaline content. The scatter of the adrenaline values was rather wide and the administration of the Rauwolfia alkaloids did not produce clear-cut changes.

In the experiments of INNES, KRAYER, and WAUD[14] the reserpine-type Rauwolfia alkaloids increased the heart rate and shortened the functional refractory period of atrioventricular (A–V) transmission and A–V propagation time in the dog heart-lung preparation. The quantitative difference in potency between Raunescine and Isoraunescine, mentioned above, was also noticed. α-Yohimbine which had no such effects did not decrease the noradrenaline content in our experiments. The assumption therefore, that the transient cardioacceleration and facilitation of A–V transmission is due to the release of noradrenaline from the heart, is further supported by our findings on the rat heart.

[1] This work was supported by a grant from the U. S. Public Health Service (H-2205) and by funds from the Eugene Higgins Trust.

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[6] A. CARLSSON, E. ROSENGREN, Å. BERTLER, and J. NILSSON, in *Psychotropic Drugs* (Ed. by GARATTINI and GHETTI, Elsevier, Amsterdam 1957), p. 363.
[7] O. KRAYER and M. K. PAASONEN, Acta physiol. scand. *42*, Suppl. *145*, 88 (1957).
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[10] J. H. BURN and M. J. RAND, Brit. med. J. *1*, 903 (1958).
[11] U. S. v. EULER, *Noradrenaline* (C. C. Thomas, Springfield, Ill. 1956), p. 64.
[12] N. T. KÄRKI, Acta physiol. scand. *39*, Suppl. *132*, 1 (1956).
[13] N. J. BEIN, Pharmacol. Rev. *8*, 435 (1956).
[14] I. R. INNES, O. KRAYER, and D. R. WAUD, J. Pharmacol., in press (1958).

According to Brodie *et al.*[15,16] only Rauwolfia alkaloids with sedative action are able to release 5-hydroxytryptamine and noradrenaline from brain tissue. Isoraunescine in our experiments was practically without sedative action but, nevertheless, able to deplete noradrenaline from the rat heart. However, a higher dose of isoraunescine than raunescine seemed necessary to produce the effect. In the brain, too, 50 mg/kg of isoraunescine lowered the concentration of noradrenaline less than 5 mg/kg of raunescine[17]. The fact that α-yohimbine was ineffective in the experiments reported is in keeping with the finding[18,19] that it does not release noradrenaline from the rat intestine, the tissue most sensitive to depletion of noradrenaline by the reserpine-type Rauwolfia alkaloids.

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Department of Pharmacology, Harvard Medical School, Boston (Mass.), September 11, 1958.

Zusammenfassung

Die chemisch verwandten Rauwolfia-Alkaloide Raunescin, Isoraunescin, Deserpidin, Rescinnamin und Reserpin verursachen einen starken Abfall des Noradrenalingehaltes des Rattenherzens, wenn sie intraperitoneal in einer Dosis von 5 mg/kg gegeben werden. Der Adrenalingehalt wird nicht deutlich oder einheitlich verändert. α-Yohimbin, das chemisch zu einer andern Gruppe gehört, hat keine Wirkung auf den Noradrenalingehalt des Herzens.

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[16] P. A. Shore and B. B. Brodie, in *Psychotropic Drugs* (Ed. by Garattini and Ghetti, Elsevier, Amsterdam 1957), p. 442.

[17] M. K. Paasonen and P. B. Dews, Brit. J. Pharmacol. *13*, 84 (1958).

[18] M. K. Paasonen and N. T. Kärki, Acta endocr., in press (1958).

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Effects of Different Vegetable Fats on the Cardiac Damage Caused by Pyridoxine Deficiency

Hypertrophy of the heart[1] and a reduction in cardiac transamination[2] are exhibited by animals when vitamin B_6 is lacking from their diet, the former being attributed to hypertension caused by lack of pyridoxine, while the latter is basically due to the vitamin in the form of pyridoxalphosphate being the prosthetic group of transaminases.

Such a picture is remarkably similar to the one observed in human arteriosclerotic forms, whose pathogenesis appears to be heavily affected by vitamin B_6[3] through the relations existing between the latter and lipid metabolism, notably that of the so-called essential fatty acids. It has been established, indeed, that, while a lack of vitamin B_6 tends to increase the severity of the Burr syndrome[4], the administration of non-saturated fatty acids during a diet lacking in pyridoxine will either delay the onset of the hypovitaminosis or mitigate its symptomatology[5].

We were induced by the foregoing considerations to undertake a program aimed to assess the actions exerted by a variety of vegetable fats, all with varying contents of essential fatty acids, upon (1) the size, (2) lipid contents, and (3) pyridoxal phosphate contents of the heart, our experiments being performed on rats submitted to a diet lacking in vitamin B_6.

We used a total of 144 animals averaging in weight from 120 to 150 g, divided into twelve groups of twelve subjects each, on a diet with no vitamin B_6, of known composition to which 25% of a vegetable fat had been added.

The subjects received as much of the above diet as they could eat during a four-week period, at the end of which they were bled to death, their hearts then being removed under aseptic conditions, weighed and tested for total-lipids[6] and pyridoxalphosphate contents. The latter substance was determined by the method developed by Boxer *et al.*[7] using *S. faecalis* as the test germ.

The iodine value of each fat used was determined by the Hanus method as described in U.S.P. XV[8].

Our results are tabulated in Tables I and II.

Table I.—Iodine Values of Fats used (Mean values ± S.E.)

Fat	Iodine Value
Coconut Oil	10·2 ± 0·4
Cocoa Butter	36·5 ± 2·1
Olive Oil	81·9 ± 3·4
Peanut Oil	93·8 ± 6·0
Cottonseed Oil	105·3 ± 8·8
Sesame Oil	107·1 ± 9·2
Corn Oil	123·8 ± 11·5
Wheat Germ Oil	125·2 ± 10·8
Soybean Oil	132·3 ± 12·1
Linseed Oil	177·6 ± 13·4

From the foregoing data it appears that the greatest degree of heart enlargement is obtained from cocoa butter and coconut oil, the slightest from soybean and linseed oils, and that intermediate degrees of hypertrophy are determined by the other fats tested. It may be noted that the order in which the fats are classed according to their iodine values has been maintained, as the lower the iodine value of the fat administered, the greater was the increase in heart weight, and *vice versa*, the only exception being cottonseed oil, whose hypertrophying effect appears to be less than that of corn oil although its iodine value is lower.

Generally, the same considerations apply to the lipid contents of the heart tissue.

On the contrary, the pyridoxalphosphate contents was highest in such subjects as were administered soybean or wheat germ oil and lowest when olive oil was added to the diet. Starting from cottonseed oil, indeed, the effect upon the coenzymic contents of the heart tissue gradually

[1] L. R. C. Agnew, Proc. Soc. exp. Biol. Med., N. Y. *90*, 452 (1955).

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[6] W. O. Fenn, J. biol. Chem. *128*, 297 (1939).

[7] G. E. Boxer, M. P. Pruss, and R. S. Goodhart, J. Nutr. *63*, 623 (1957).

[8] U. S. Pharmacopoeia XV (Mack Publ. Co., Easton 1955), p. 896.

Table II.—Variations in Weight, Lipid, and Pyridoxalphosphate Contents in the Heart of Rats (Mean value ± S. E.)

Diet Type	Fat Administered (Diet 'C')	Heart Weight (mg/100 g body weight)	Total Lipids (g%)	Pyridoxalphosphate (µg/100 g)
A	—	324·8 ± 29·5	1·667 ± 0·104	108·6 ± 8·1
B	—	418·5 ± 38·1	2·194 ± 0·178	41·7 ± 3·5
C	Coconut Oil	513·2 ± 42·7	2·838 ± 0·261	16·0 ± 1.3
C	Cocoa Butter	497·5 ± 41·4	2·751 ± 0·246	14·8 ± 1·1
C	Olive Oil	490·8 ± 44.3	2·696 ± 0·225	12·1 ± 0·7
C	Peanut Oil	479·3 ± 38.6	2·655 ± 0·218	14·6 ± 1·2
C	Cottonseed Oil	450·7 ± 36·2	2·618 ± 0·234	24·2 ± 2·2
C	Sesame Oil	461·4 ± 40·7	2·573 ± 0·226	21·5 ± 1·8
C	Corn Oil	454·9 ± 41·5	2·546 ± 0·198	22·9 ± 2·1
C	Wheat Germ Oil	441·6 ± 39.2	2·470 ± 0·183	27·4 ± 2·5
C	Soybean Oil	433·6 ± 41·4	2·484 ± 0·232	29·5 ± 2·7
C	Linseed Oil	428·5 ± 37·4	2·469 ± 0·177	32·2 ± 1·9

Diet 'A' – Devitaminized casein, 16% – Corn Oil, 15% – Vitaminized lactose, 4%* – Salt mixture IV, 4% – sucrose to make 100.
Diet 'B' – Same as 'A' but minus vitamin B_6 in vitaminic supplement.
Diet 'C' – Same as 'B' plus 25% vegetable fat.

* 100 g vitaminized lactose contain: thiamine mononitrate, riboflavine, and pyridoxine hydrochloride, each 12·5 mg – calcium pantothenate, p-aminobenzoic acid, each 50 mg – nicotinamide, 62·5 mg – biothin, folic acid, each 2·5 mg – vitamin A acetate (cryst.), 25000 U. – vitamin D 2, 7500 U. – choline chloride, 5 g – inositol, 2·5 g.

decreases with no evident, direct relationship to the iodine values of the oils used.

To sum up, while heart hypertrophy and lipid contents appear to be evidently related to the non-saturated fatty acid contents of the oils added to the diet, no similar relation exists in respect of pyridoxalphosphate concentration. Consequently, if the effect exerted by alimentary fats upon heart size and lipid contents may be attributed to non-saturated fatty acids being more easily metabolized than saturated ones, the same reasoning does not apply to the action exerted by the very same fats on the coenzyme, whose variations seem to be explainable otherwise.

One of the possible interpretations of the latter phenomenon is related to the oleic acid contents of the fat used, as this acid is known to aggravate the effects of a lack of vitamin B_6[9]. This would account for the severer depletion of pyridoxalphosphate in the heart tissue of such rats as were administered olive oil, wherein oleic acid amounts to 85% of the aggregate fatty acid contents. In addition to this, oleic acid promotes the absorption of alimentary cholesterol by the intestine to a substantial extent[10], thereby further increasing such already heavy cholesterol concentrations as are determined in the blood by lipid diets.

Such a view appears to be supported by the fact that the decrease in pyridoxalphosphate concentrations determined by peanut oil (wherein oleic acid amounts to 57·2% of total fatty acids) is only slightly less than the one determined by olive oil, as well as by the fact that a greater effect is developed by cocoa butter than by coconut oil, the former featuring a higher iodine value but also a greater amount of oleic acid. The same remark applies to cottonseed oil in respect of both sesame and corn oils.

Finally, a considerably important factor appears to be the linoleic contents of the fats used, for the fact should be stressed that linseed oil (highest iodine value among all fats tested) appears to lower the pyridoxalphosphate contents to a greater extent than do cottonseed, wheat germ or soybean oils – all of which have a higher linoleic-acid contents although their iodine values are lower.

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Farmavigor S. p. A. Milano, Research Department, October 9, 1958.

Riassunto

L'effetto di diete contenenti un eccesso di grassi vegetali e carenti di vitamina B_6 è stato studiato dal punto di vista del contenuto di lipidi e di piridossalfosfato nel tessuto cardiaco. Dei dieci grassi vegetali presi in esame il massimo aumento dei lipidi cardiaci si verifica per il burro di cacao e per l'olio di cocco, i più saturi di tutti i grassi usati.

Per quanto riguarda le concentrazioni cardiache di piridossalfosfato la massima diminuzione si registra per l'olio d'oliva, mentre la diminuzione minima si registra per l'olio di germe di grano e per l'olio di soia. Vengono brevemente discusse, dal punto di vista del grado di insaturazione e del contenuto di acidi grassi, le possibili cause di questi risultati.

[9] P. S. Sarma, E. E. Snell, and C. A. Elvehjem, J. Nutr. *33*, 21 (1947).
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Appetite Stimulating Effect of Barbiturate-Induced Therapeutic Sleep

In our previous papers[1,2] we gave evidence of an increased food intake as one of the characteristic metabolic peculiarities of therapeutic sleep (T. S.). An attempt at a more detailed analysis of this phenomenon was made in subsequent work by comparing caloric balances in a total of 50 patients (43 with peptic ulceration, 7 with various neurotic states), treated by T. S., induced by barbiturates

[1] E. Kuhn and J. Mašek, Dtsch. Ges.-Wes. *11*, 577 (1956).
[2] E. Kuhn and J. Mašek, Acta Inst. Aliment. hum. Pragae *1*, 275 (1955).

(Amytal, Amobarbital, Dormiphen; 38 patients; average dose 0·26 g/day), chlorpromazine (50–200 mg/day; 7 patients), and by conditioned sleep (5 patients). The standard procedure and results were published in detail elsewhere[3].

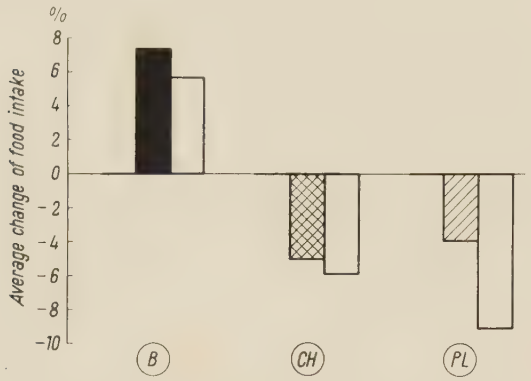

Fig. 1.—Changes of average food intake during period of sleep (black or shaded columns) and during 2nd control period (white columns) are expressed as percentages of the initial caloric intake during 1st control period (—)
B: barbiturate-induced sleep, CH: Chlorpromazine-induced sleep, Pl: conditioned sleep

Results: Changes in the food intake (Fig. 1) indicate clearly the difference between the group of patients where sleep was induced by barbiturate (average increase by 7·85%), chlorpromazine (average decrease by 4·99%), and conditioned sleep (average decrease by 3·75%). The analysis of dispersal made by means of the non-parameter test (Table) indicates that the differences of the average daily caloric intake during the investigation periods in all groups are statistically highly significant. The graphic presentation of the changes of food intake during barbiturate-induced sleep shows an even more significant difference (Fig. 2) if the average maximal caloric increase recorded during 3–5 days (on end) of the sleep period is used for comparison. We were, however, unable to reveal any relation between the caloric increment and the size of the administered dose of barbiturate.

Fig. 2

Discussion: Among various papers dealing with the neurogenic regulation of appetite, the work of Brobeck *et al.*[4] appears important in explaining the mechanism of the changes observed. These authors, while investigating changes of electrical activity in various parts of the hypothalamus under the influence of amphetamine and its derivatives, noted a secondary finding during light anesthesia: barbiturate spindles from the hypothalamus with the exception of the ventromedial nuclei and from the neighbouring parts of the diencephalon and mesencephalon, i.e. from areas the electrical activity of which is not influenced by amphetamine preparations. This finding supplements their previous empirical observation of an increased food intake in rats with lesions of the ventromedial nucleus when recovering from barbiturate anesthesia[5]. As far as clinical work is concerned, we should like to draw attention to Baumann's trial to apply T. S. treatment in certain types of diabetes[6], the experience of Hunter and Greenberg[7] who observed deviations of carbohydrate metabolism associated with the habitual use of barbiturates, as well as to our own findings concerning the influence of barbiturates on the behaviour of glycemia of experimental subjects during 5 h periods on fasting, etc. As appears from Mark's communication[8], who observed that acetylation was inhibited to a different degree according to the type of barbiturate used, we may assume that the carbohydrate metabolism may be influenced in a different manner by different types of barbiturate used.

Kind of Therapeutic Sleep	Experimental Period	
	1st Control: Sleep	1st Control: 2nd Control
B	95·50**	155·36**
CH	9·18**	10·70**
Conditioned	17·39**	21·65**

** Values are statistically significant at the 1% level.

In our original explanation of the increased food intake under the influence of barbiturate-induced sleep[1,2] we concentrated our attention on the regulatory action of the central factor, where a predominance of inhibition changes the mutual relationship between cortex and subcortical regions, particularly by eliminating its inhibitory action on hypothalamic nuclei which are an important part of the functional structure of the alimentary centre as conceived by Pavlov[9]. The finding of Brobeck *et al.*, recorded but not analysed in greater detail, of a certain relation of anesthesia to the regulation of food intake does not rule out a possible more specific connection of a certain type of barbiturates and the controlling function of the lateral or medial mechanism. This can manifest itself as activation of the lateral or inhibition of the ventromedial nucleus. We are, however, aware of the fact that apart from producing purely nervous phenomena by the pharmacological action of hypnotics, these substances may directly influence the metabolic activity on an enzymatic level and interfere with the carbohydrate metabolism. These mechanisms may regulate the biochemical homeostasis and act at the same time on neurogenic factors. The structural similarity of the barbituric acid molecule and alloxan as well as the hypoglycaemic effect of some barbiturates we referred to, does not rule out the possible participation of insulin in the development of hyper-

[3] E. Kuhn, Čas. lék. čes., in press.

[4] J. R. Brobeck, S. Larsson, and E. Reyes, J. Physiol. (London) *132*, 538 (1956).

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[8] B. H. Marks, Science *123*, 332 (1956).

[9] I. P. Pavlov, *Collected Works*, vol. 3 (Moscow 1951), p. 147.

phagia. A differentiation of these individual mechanisms of the action of barbiturates – the nervous and humoral component of the regulation of food intake, based hitherto assembled clinical results, is not yet possible.

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Zusammenfassung

Im Gegensatz zum Chlorpromazin- und bedingt reflektorischen Heilschlaf löst die Medikation von kleinen Dosen kurzwirkender Barbiturate während der 14tägigen Schlafperiode bei Geschwürkranken und Neurotikern eine zeitweilige Hyperphagie verschiedenen Grades aus. Der relative Anteil von einzelnen mitbeteiligten Mechanismen wird diskutiert.

PRO EXPERIMENTIS

Ein Nomogramm zur Kombination mehrerer Ergebniswahrscheinlichkeiten

Für die zusammenfassende Beurteilung der gemeinsamen Wahrscheinlichkeit mehrerer voneinander unabhängiger Versuchsergebnisse gibt es verschiedene methodische Möglichkeiten[1,2], von denen die Kombination mittels der χ^2-Werte nach Fisher[3] wohl die unter den gewöhnlichen Umständen vorteilhafteste[1] und deshalb auch die verbreitetste[4] sein dürfte. Sie beruht darauf, dass die Grösse $-2 \sum_1^k \log_e P_i$ wie χ^2 mit $2k$ Freiheitsgraden verteilt und daher exakt testbar ist.

Das angeführte Nomogramm gestattet nun die schnelle und hinreichend genaue graphische Bestimmung der kombinierten Totalwahrscheinlichkeit P_t bei der Zusammenfassung von 2, 3 oder 4 Versuchen. Der Ablesungsvorgang ergibt sich aus der Rechentafel selbst, bzw. ist aus den beigefügten Schemen zu erkennen.

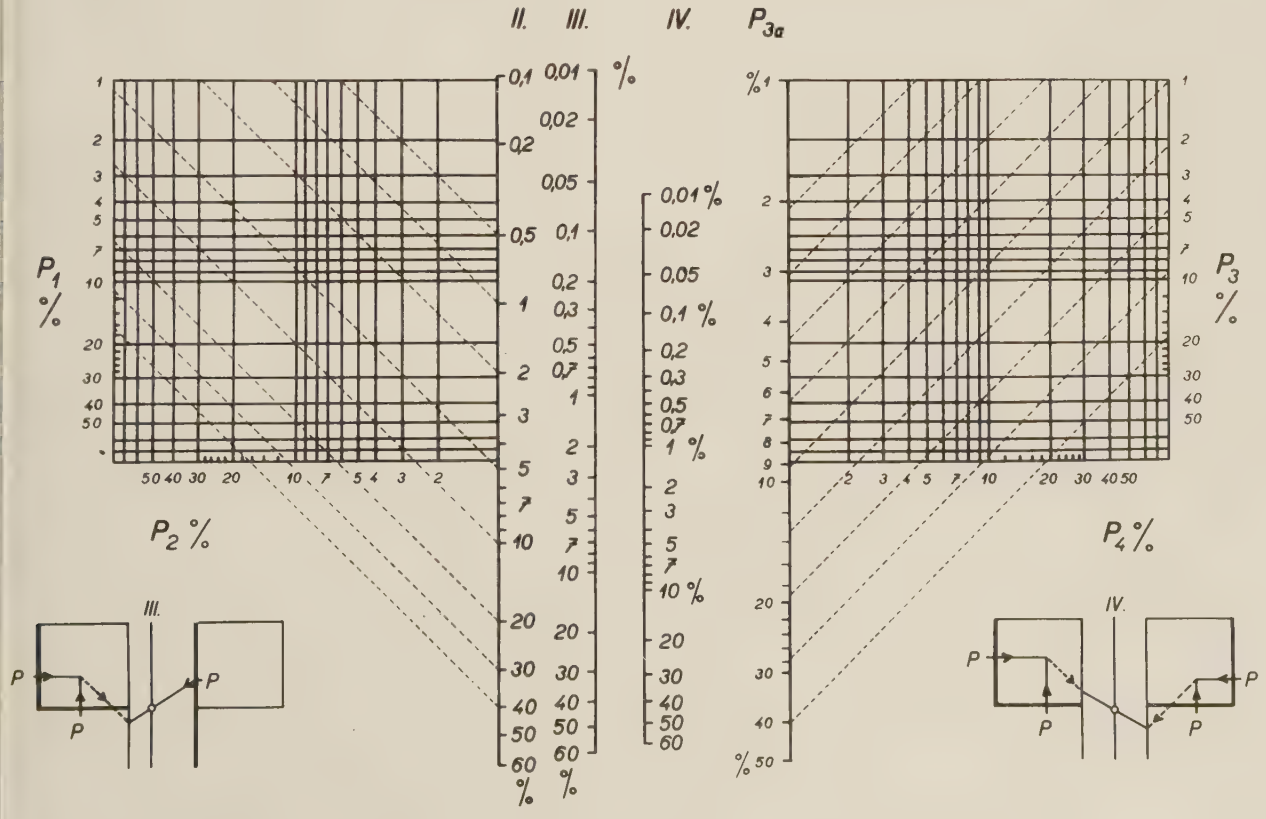
Im Falle zweier Versuche genügt die linke Bildhälfte: man geht mit P_1 und P_2 in die linke Netztafel ein und erhält das gesuchte P_t mittels deren Schnittpunktes, bzw. genauer durch dessen Verschiebung parallel zu den gestrichelten Leitlinien bis auf die *Resultatleiter II*.

Im Falle dreier Versuche wird die Leiter II zur Dreh- oder Zapfenlinie[5]: von ihr aus wird eine Ablesegerade durch den Wert für P_3 auf Leiter $P3a$ (nicht P_3!) gelegt, deren Schnittpunkt mit der *Resultatleiter III* das gesuchte P_t ergibt.

Im Falle von vier Versuchen geht man wie bei II, aber beiderseits symmetrisch von P_1 und P_2 links, bzw. P_3 und P_4 rechts aus vor, wobei zu beachten ist, dass nunmehr auch Leiter $P3a$ zur Zapfenlinie wird. (Ihre Skala entspricht nur dem P eines einzigen Versuches, nämlich dem des dritten von dreien, und ist daher als solche zur Ablesung des kombinierten Wertes von P_3 und P_4 allein nicht verwendbar.) Die gesuchte Totalwahrscheinlichkeit P_t wird in diesem Falle mittels einer durch die entsprechenden Punkte auf II und $3a$ gelegten Ablesegeraden auf der *Resultatleiter IV* ermittelt.

Abschliessend wird noch darauf hingewiesen, dass zwecks Vermeidung überflüssiger Dezimalstellen alle P-Werte *in Prozenten* angegeben sind und daher zum Beispiel der Ausdruck «0,1» einem Zehntelprozent, das heisst einer Wahrscheinlichkeit von 0,001 entspricht.

[1] A. Birnbaum, J. Amer. stat. Ass. *49*, 559 (1954).
[2] K. Pätau, Biol. Zbl. *63*, 152 (1943).
[3] R. A. Fisher, *Statistical Methods for Research Workers* (Edinburgh 1934).
[4] G. W. Snedecor, *Statistical Methods*, 5th Ed. (Ames 1956).
[5] P. Luckey, *Nomographie* (Leipzig 1953).



Herrn D. Lauko sei an dieser Stelle für die technische Ausführung aufrichtiger Dank ausgesprochen.

R. Štukovsky

Endokrinologisches Institut der Slowakischen Akademie der Wissenschaften, Bratislava (Tschechoslowakei), 22. August 1958.

Summary

A nomograph is presented, which gives (according to Fisher's formula) the pooled probabilities for the combination of two, three, and four independent experiments respectively.

PRO EXPERIMENTIS

A Method for the Separation and Estimation of Catechol Amines in Urine

Adrenaline, noradrenaline, and a third catecholamine, namely, hydroxytyramine are constituents of normal urine[1–3]. While biological methods are available for determination of catecholamines in blood and urine, these methods have obvious limitations for routine work and can only be employed for the determination of the pharmacologically active hormones, adrenaline and noradrenaline. A differential fluorometric method[4] has been used in the past for determination of all three catecholamines. In this method the catecholamines are estimated by a combination of the fluorometric methods of Weil-Malherbe and Bone[5] and of von Euler and Floding[6]. The former method estimates the sum of the three catecholamines. Adrenaline and noradrenaline are estimated separately by the latter, and hydroxytyramine is obtained by the difference in results between the two methods.

It seemed desirable to develop a method for simultaneous estimation of all three catecholamines. In previous experiments[7,8], it was shown that it is possible to acetylate catecholamines quantitatively in biological tissue and urine. These acetylated derivatives of adrenaline, noradrenaline, and hydroxytyramine have been shown to be stable, whereas the free compounds are known for their instability. In the procedure described below we acetylated the catecholamines in urine and separated the acetylated derivatives by paper chromatography.

Urine samples were acidified to pH 2 and hydrolyzed by refluxing at 100°C for 30 min. After cooling to room temperature, the urine sample was extracted four times with an equal volume of ethylacetate. The organic phase contained all the acidic catechols which could interfere with the separation of the catechol amines. The aqueous urine extract was adjusted to pH 4 by adding 0·5 *n* NaOH while stirring. The catechol amines in the extract were acetylated by adding 5 g $NaHCO_3$ and 2·5 cm^3 acetic anhydride in four equal portions with constant stirring until foaming ceased. Methylene chloride (50 ml) was added and stirring was continued for another 10 min. The mixture was transferred to a separatory funnel and the organic phase removed. The water was extracted three times with 50 ml of methylene chloride and the combined methylene chloride extracts were counter washed with 50 ml of water filtered through Na_2SO_4 and evaporated in a vacuum. The dry extract was dissolved in 0·5 ml of methyl alcohol and applied to Whatman No. 1 paper for chromatographic separation in the Bush system 'C'[9] using as solvents: toluene, ethyl acetate, methyl alcohol, and water in the ratio 10:1:5:5.

Table I
Comparison of R_f Values obtained after Paper Chromatography of Free Catechol Amines and their Triacetate Derivatives

Catecholamine	R_f Values	
	Free Compound*	Triacetate Derivative**
Noradrenaline	0·15	0·12
Adrenaline	0·28	0·50
Hydroxytyramine	0·33	0·82

* Solvent System: Phenol/HCl.
** Solvent System: Toluene: ethyl acetate: methyl alcohol.

Ascending chromatograms were developed after 24 h. The control area, which contained 100 γ of adrenaline acetate, noradrenaline acetate, and hydroxytyramine acetate, was developed by spraying first with 10% KOH in MeOH:H_2O (1:1) and then with a freshly prepared mixture of 1% $FeCl_3$ and 2% $K_3Fe(CN)_6$. Adrenaline acetate, noradrenaline acetate, and hydroxytyramine acetate were assumed to be opposite their control spots. These areas were cut out and a mixture of ethylenediamine and ethylenediamine hydrochloride was added to corresponding paper strips. A one step reaction was carried out by shaking and heating this mixture at 50°C, resulting in hydrolysis of the acetate derivative and condensation of the free compound with ethylenediamine. The fluorescent condensation product was extracted into isobutanol and a quantitative fluorometric determination using the Weil-Malherbe[5] method was carried out.

A comparison of the R_f values (see Table I), obtained from paper chromatography separation of the acetylated catecholamine derivatives and the free compounds, shows that an effective separation can be carried out by the use of this method. Another advantage of the method is the elimination of the adsorption of the catecholamines on alumina. Adsorption on alumina, which was an essential step in previous procedures, is known to be only a semi-quantitative operation.

A preliminary study of the acetylation method for the separation and determination of catecholamines has been completed on the urines of ten psychiatric patients with various diagnoses who were receiving different treatments. The results (see Table II) demonstrate a wide inter-subject variability among the psychiatric patients studied. Values obtained by this method are higher than those obtained by bioassay[10] or by specific fluorometric methods[6]. This may be the result of the elimination of alumina adsorption or may occur because of non-specificity of the condensation with ethylenediamine.

[1] P. Holtz *et al.*, Arch. exp. Path. Pharmakol. *200*, 356 (1942).
[2] U. S. von Euler *et al.*, Biochem. J. *49*, 655 (1951).
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[7] M. Hogopian, to be published.
[8] M. Goldstein, M. Hogopian, and R. I. Dorfman, IV. Int. Biochem. Congress, Vienna 1958.
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Table II
Excretion of Noradrenaline, Adrenaline, and Hydroxytyramine in Various Psychiatric Patients

Subject	Noradrenaline (μg/100 mg creatinine)	Adrenaline (μg/100 mg creatinine)	Hydroxytyramine (μg/100 mg creatinine)
1	2·80	0·60	39·00
2	4·90	1·10	28·90
3	2·20	0·30	24·50
4	1·90	0·50	26·00
5	2·45	0·38	24·80
6	7·90	1·32	39·30
7	6·85	0·95	31·00
8	8·00	1·67	46·00
9	6·25	1·50	52·50
10	4·10	0·02	36·00

Further specificity can be obtained by using a double-label technique. Urine extracts can be acetylated with tritium labeled acetic anhydride to convert adrenaline, noradrenaline, and hydroxytyramine quantitatively to the tritium labeled triacetate. A measured amount of authentic triacetate adrenaline-C^{14}, triacetate noradrenaline-C^{14}, and triacetate hydroxytyramine-C^{14} is added to each sample and the double labeled catecholamines are identified and purified by paper chromatography. The tritium and carbon^{14} content of the purified catecholamines is assayed by simultaneous counting in a liquid scintillation spectrometer. The amount of adrenaline, noradrenaline, and hydroxytyramine in the original extract can be calculated from the determination of the amount of carbon^{14} indicator lost during the purification, the yield of tritium radioactivity, and the specific activity of the tritium labeled acetic anhydride.

M. Goldstein, A. J. Friedhoff, and C. Simmons

New York University College of Medicine, Department of Psychiatry and Neurology, Psychopharmacology Research Unit, New York, November 3, 1958.

Zusammenfassung

Es wird eine neue Methode zur Bestimmung der Katecholamine im Harn beschrieben. Die Katecholamine werden im Harn acetyliert und die Acetylderivate papierchromatographisch im Bush-«C»-System getrennt. Nach erfolgter Trennung werden die einzelnen Katecholamine mit Äthylendiamin kondensiert und fluorimetrisch untersucht. Eine Spezifitätserhöhung der Methode durch Anwendung doppelt markierter Isotopen wird in Betracht gezogen.

Informations - Informationen - Informazioni - Notes

THEORIA

Thermal Polymerization of Amino Acids and a Theory of Biochemical Origins[1]

By S.W. Fox, Kaoru Harada, and A. Vegotsky[2]

The possibility of synthesizing peptides and perhaps protein (Fox and Middlebrook[3]) by simple heating of unsubstituted amino acids is one which has not been favored by chemical experience (Katchalski[4], Noguchi and Hayakawa[5], Curphey[6], Meggy[7], Bamford, Elliott, and Hanby[8]). When this goal was approached as a theoretical problem in prebiological molecular evolution, it became possible to visualize how the energetic barrier for the synthesis of protein could be overcome thermally (Fox, Johnson, and Vegotsky[9]), and how precise ordering of residues might result from selective influences of the reactant amino acids themselves (Fox[10]). The experiments which have resulted indicate that it is possible to prepare copolymeric peptides thermally if aspartic acid or glutamic acid is a reactant. It is furthermore possible, by using a considerable molar excess of both aspartic acid and glutamic acid, to copolymerize the eighteen common amino acids into a *proteinoid* which, after being hydrolyzed, reveals on paper chromatography the same qualitative composition as casein and other proteins.

In considering this knowledge in detail, attention should first be focussed on the chemistry of each of the dicarboxylic amino acids. It has long been known that heating glutamic acid alone yields the inner lactam, pyrrolidonecarboxylic acid (pyroglutamic acid). In this research it has been learned that heating glutamic acid with each of a number of other amino acids produces a copolymeric linear peptide when the temperature of reaction is 170°C for 3 h (Harada and Fox[11]). The polymer of glutamic acid and glycine has a mean chain weight of 10,000 to 20,000 after dialysis. During such reactions, the glutamic acid is converted to pyroglutamic acid which is molten in the presence of other materials at 170°C, and functions therefore as a solvent. The glutamic acid and pyroglutamic acid probably contribute also as acid catalysts. In addition, comparative yields suggest that the pyroglutamic acid is the more reactive species.

[1] Presented at a symposium on Biochemical Origins at the 133rd meeting of the American Chemical Society, San Francisco, April 17 1958. The research has been aided by Grant C-3971 of the National Institutes of Health, U. S. Public Health Service, Grant G-4566 of the National Science Foundation, and by the General Foods Corporation. Contribution No. 109 of the Oceanographic Institute of The Florida State University.

[2] The Oceanographic Institute and Department of Chemistry of The Florida State University, Tallahassee.

[3] S. W. Fox and M. Middlebrook, Fed. Proc. *13*, 211 (1954).

[4] E. Katchalski, Adv. Protein Chem. *6*, 123 (1951).

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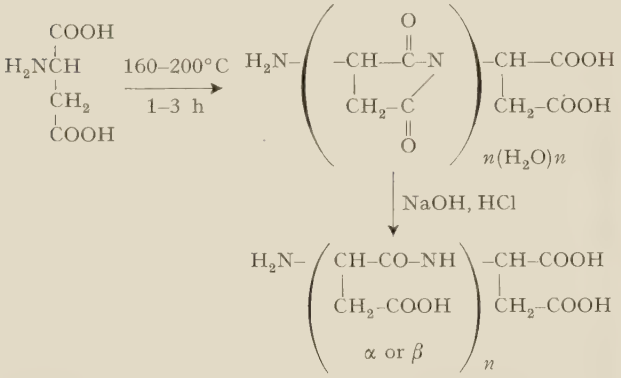
[11] K. Harada and S. W. Fox, J. Amer. chem. Soc. *80*, 2694 (1958).

Relative Proportions of Glutamic Acid in Total Composition and N-Terminal Position in Thermal Polymers of Glutamic Acid and Aspartic Acid

Mols aspartic acid in reaction mixture	Mols glutamic acid in reaction mixture	Proportion of glutamic acid in whole polymer (A)	Proportion of glutamic acid in N-terminal position (B)	B/A
0·01	0·01	29%	59%	2·0
0·02	0·01	19%	54%	2·8
0·03	0·01	15%	54%	3·6

The heating of aspartic acid does not lead to a liquid product below 200°C, but it can also copolymerize with other amino acids. The copolymers with aspartic acid do not include as large proportions of neutral and basic amino acids as do the copolymers with glutamic acid.



An unique feature of the thermal polymerization of aspartic acid is that it yields a polyimide (Kovacs and Koenyves[12], Vegotsky, Harada, and Fox[13]). This polyimide can be gently split at each of the imide linkages by excess dilute cold alkali to yield a true peptide. The tendency of aspartic acid to copolymerize appears to be greater when heated with glutamic acid and this is probably explainable on the basis that all of the aspartic acid and glutamic acid is in a clear liquid at 170°C.

The copolymerization of glutamic acid and aspartic acid proceeds with exceptional facility. In this combination was first observed a salient relationship – the amino acids arrange themselves in a nonrandom style. This is illustrated in the experiment detailed in the Table.

The analyses were performed by hydrolysis of the whole polymer, DNPylation, and estimation of the DNP-amino acids. The N-terminal amino acids were determined on the hydrolyzed DNP-polymers. The results show that the proportion of glutamic acid in the N-terminal position is from 2 to $3^1/_2$ times as great as in the total composition. This substantiates the original working hypothesis mentioned at the outset and answers in principle the question of how precise order might have arisen in primordial protein before there were enzymes or nucleic acids on the earth to guide the order.

By the time this much knowledge had accumulated, the original goal of synthesis of protein was more precisely defined as the synthesis of polymeric peptide material which contains all of the eighteen amino acids common to protein (Fox[10]), without regard to configuration of the amino acid residues (Fox, Johnson, and Vegotsky[9]). Although other working premises can be adopted (Akabori, Okawa, and Sato[14]), material of this description would be qualitatively indistinguishable from current protein and perhaps more like original protein than current protein. Part of the reason for believing that the first protein was so like current protein is the concept of the 'unity of biochemistry' (Kluyver and Van Niel[15]) which has been found to apply to proteins as well as to other substances (Fox[10], Fox[16]).

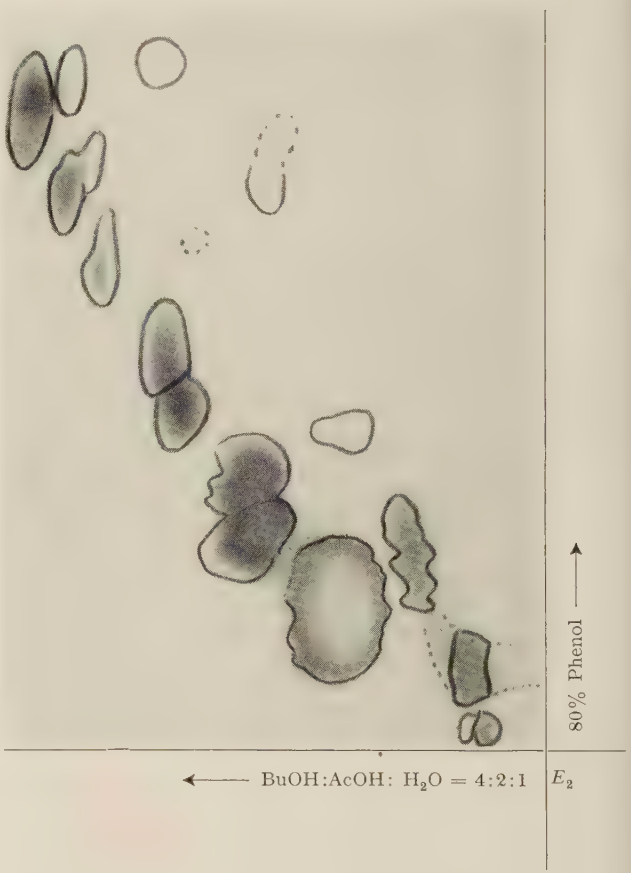


Fig. 1. Two-dimensional chromatogram of hydrolyzate of proteinoid

Attempts to pyropolymerize all the common amino acids have been made from time to time in this program. These attempts were based usually on unimolar ratios of amino acids. After the effect of a dominant glutamic acid-aspartic acid ratio, as well as the unique copolymerization effects of each were recognized, such attempts were again repeated with these two amino acids in large excess. The

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[13] A. Vegotsky, K. Harada, and S. W. Fox, J. Amer. chem. Soc. *80*, 3361 (1958).

[14] S. Akabori, K. Okawa, and M. Sato, Bull. chem. Soc. Japan *29*, 608 (1956).

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[16] S. W. Fox, J. chem. Education *34*, 472 (1957).

results were immediately successful (Fox and Harada[17]) and each of a series of more than twenty subsequent trials has likewise been successful. Renewed attempts to obtain such polymers with unimolar ratios of the dicarboxylic amino acids reveal that even if peptide is formed, the simultaneous formation of unwanted products such as diketopiperazines is so dominant as to make isolation of the peptides difficult at best. Critical need for high proportions of glutamic acid and aspartic acid is consistent with the principle of unity in biochemistry and the facts of (1) disproportionately high ratios of the dicarboxylic amino acids in virtually all proteins and (2) the large amounts of glutamine and asparagine in metabolic pools.

The proteinoids are typically produced simply by heating two parts of glutamic acid by weight, two parts of aspartic acid, and one part of a mixture of amino acids in equimolar proportions at 170°C for 3 h. The unreacted material and smaller molecules are removed by dialysis through a period of five days. In some cases the initial product was sterilized prior to dialysis by immersion of the dialysis sac in methanol or ethanol for 24–48 h. The product yielded a major insoluble fraction and a soluble fraction. The insoluble fraction was dissolved as the sodium salt and dialyzed. The products from this, from the originally soluble fraction, and from the runs which were sterilized were each dried, hydrolyzed, and chromatographed. The chromatograms appeared as in Figure 1 in all cases (Fox and Harada[17]). The reaction is not delicate, and many variations are possible. Glutamic acid can be replaced by glutamine. Orthophosphoric acid improves the yield and lowers the necessary temperature by typically 20°C, but the reaction proceeds successfully in the absence of phosphoric acid, in yields by weight which exceed 15%. The proteinoid replaces peptone in the broth culture of *Lactobacillus arabinosus* at a somewhat reduced rate of growth, a fact which indicates that the linkages are susceptible to the attack of bacterial proteases. The product is undoubtedly a mixture and it will be some time before it is possible to state how the amino acids are distributed among individual molecules. The product has in peptide linkage all of the common amino acids, except of course that these are optically inactive, and the hydrolyzate behaves in qualitative chromatography like that from protein. This relationship is one which resembles closely that for any two current heterologous proteins. It is significant that this proteinoid was ob-

[17] S. W. Fox and K. Harada, Science *128*, 1214 (1958).

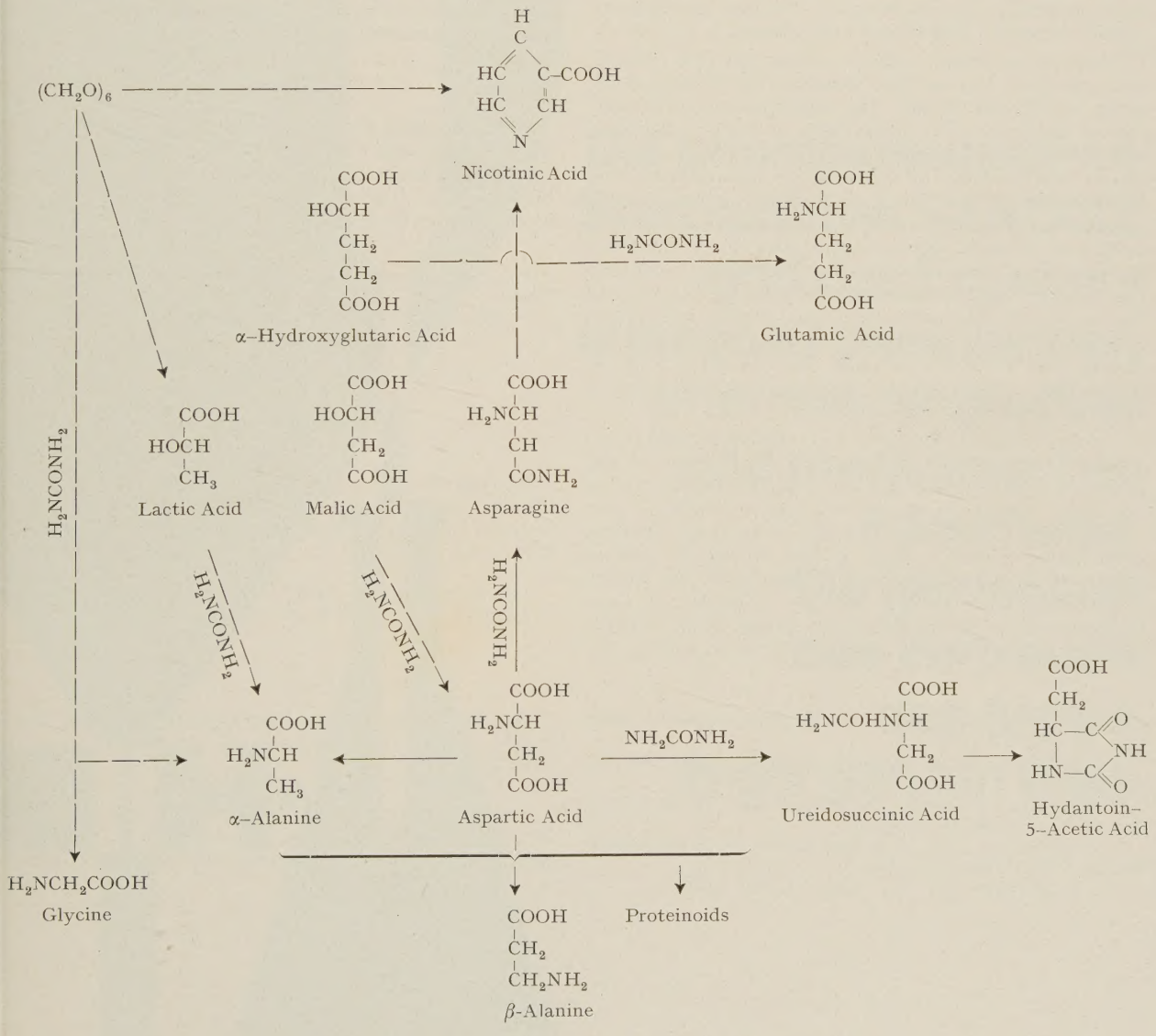

Fig. 2. Thermal pathways which resemble generalized biosynthesis

tained in a hypothetical framework of prebiological conditions. Added support for a protein-like structure of the polymer is found in positive biuret tests, infrared absorption spectra, and a mean chain weight of 4900.

Shortly after this project on thermal syntheses of peptides was begun, unexpected results under these same conditions led to other investigations (Fox, Johnson, and Middlebrook[18]). There was observed the appearance of amino acids, α- and β-alanine, not present in the original. This result suggested that our known family of amino acids may have originated by thermal conversion and rearrangement. One biochemical thing led to another and a thermal reaction flowsheet as in Figure 2 was assembled. These are some of the reactions which have been studied; the solid lines represent those for which the details have been published. It was found that aspartic acid can arise thermally from the Krebs Cycle acid, malic acid, by reaction with ammonia or urea and that aspartic acid is converted to α- and β-alanine. An unexpected product of especial interest was ureidosuccinic acid (Fox, Johnson, and Vegotsky[9]).

This partial chart includes many thermal products identical to those found in organisms, except for lack of optical activity in some. The reactions are also similar and, most strikingly, the sequences of thermal reactions resemble those of a generalized biosynthesis.

These unified results indicate in principle how the anabolic reactions and protein, somehow functioning as enzyme, could arise jointly. The appearance of ureidosuccinic acid suggests the biosynthesis of genic nucleic acid inasmuch as ureidosuccinic acid is now a recognized biological intermediate for pyrimidines (Lieberman and Kornberg[19]). From this it is possible to visualize the generation of anabolic reactions, enzymes, and genes in a reflexive chemical memory mechanism (Fox, Vegotsky, Harada, and Hoagland[20]). This picture is essentially the reverse of the gene-enzyme-reaction system of Beadle[21] *et al.* The entire integrated picture is of course incomplete, but it is brought into sharper focus by the finding that all of the common amino acids can be copolymerized under selected thermal conditions into a single product suggestive of protein.

Purely biological evidence for the thermal origin of organisms, although not conclusive, can be found in the literature. The proposal that life began in thermal waters has been offered by several biologists, notably Copeland[22]. One criticism of chemical or biological theories emphasizing thermal origins is that biological systems cannot withstand such elevated temperatures. One answer to this problem is the fact that some bacteria and algae normally inhabit nearly boiling hot springs. These, according to Copeland, are the most primitive organisms. Secondly, Greenstein and Hoyer[23], and also Hamer[24], showed that many substances inhibit the thermal coagulation of protein; notably nucleic acids do this. In attempting to understand the origin of physiological systems, it may be necessary therefore to understand the special influences of molecular interactions quite as fully as the generation of substances and reactions.

Despite increasing numbers of experimental demonstrations, more than usual uncertainty attends any interpretations in the subject matter of biochemical origins. A true understanding of origins however offers much promise of systematizing the tremendously ramified mass of biochemical knowledge and is to be sought for this reason alone.

At the most the picture which has been inferred represents essentially the way in which biochemical systems originated. At the least, it presents in relatively complete outline an internally and externally consistent picture of how biochemical systems, and by self-directed extrapolation, life (Fox[16]) could have originated, when one invokes a modulation from a hypohydrous magma to an aqueous system.

At the non-interpretative level, the studies have revealed ways and means of producing with relative ease a large variety of peptides which command interest in a number of potential applications.

Zusammenfassung

Bei Überschuss von Glutaminsäure und Asparaginsäure werden zahlreiche Aminosäuren thermisch kopolymerisiert. Das Studium der Reaktionen und Nebenreaktionen führt zu einer Theorie über den thermischen Ursprung biochemischer Systeme.

[18] S. W. Fox, J. E. Johnson, and M. Middlebrook, J. Amer. chem. Soc. *77*, 1048 (1955).

[19] I. Lieberman and A. Kornberg, Biochim. biophys. Acta *12*, 223 (1953).

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[21] G. W. Beadle, *First R. E. Dyer Lecture* (U. S. Government Printing Office, Washington 1951).

[22] J. J. Copeland, Ann. N. Y. Acad. Sci. *36*, 1 (1936).

[23] J. P. Greenstein and M. L. Hoyer, J. biol. Chem. *182*, 457 (1950).

[24] D. Hamer, Biochem. J. *56*, 610 (1954).

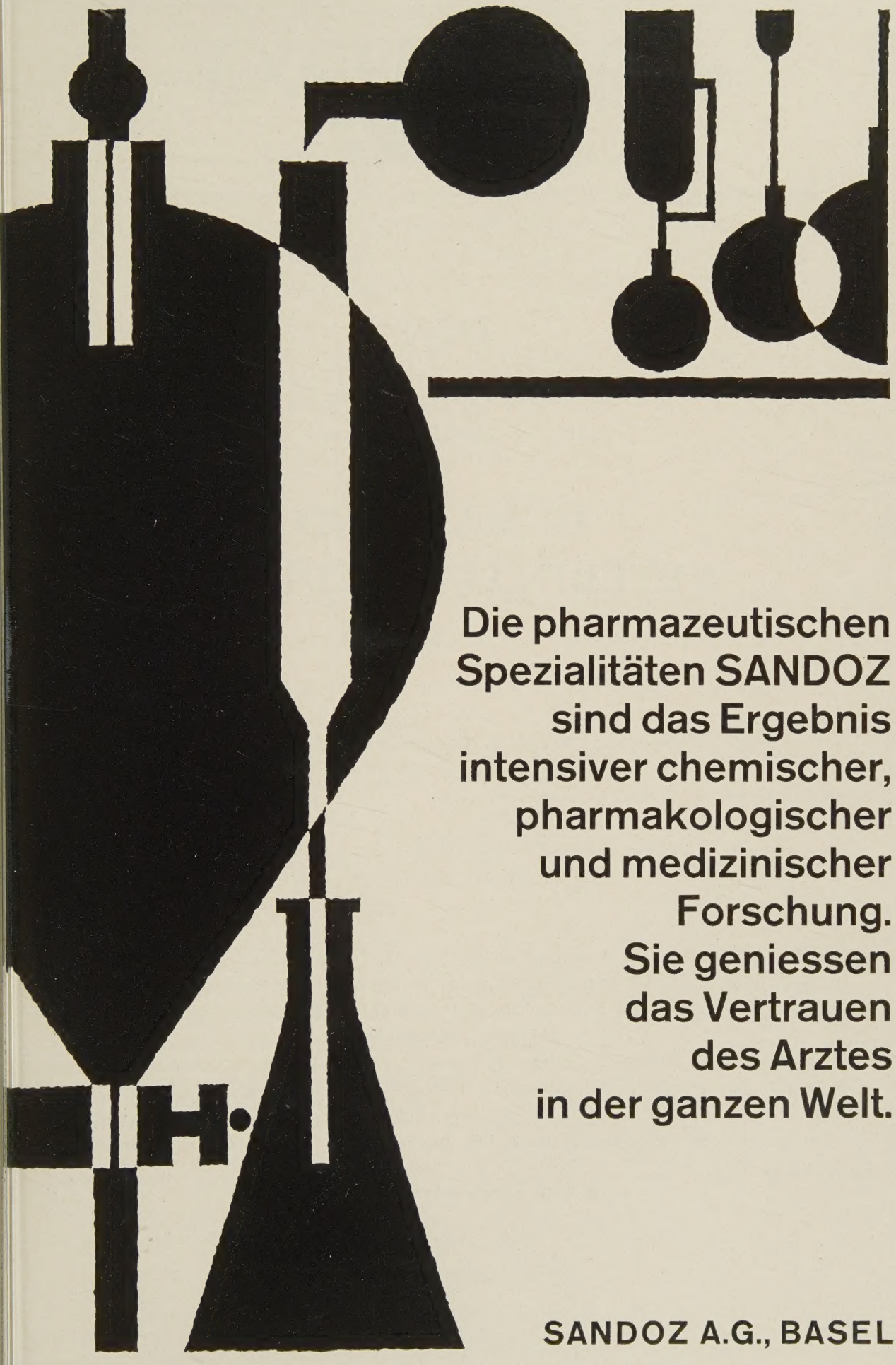
Corrigenda

Roshan J. Irani and K. Ganapathi: *The Effect of Glycerol on the Biosynthesis of Benzylpenicillin by the Washed Cells of Penicillium chrysogenum*, Exper. *14*, fasc. 9, 329 (1958).

The third entry in Table III should read: PA (0·05%) + glycerol (1%) + KCN (0·005 *M*).

H. Grossfeld: *Fat Formation and Glycolysis in Tissue Culture. Action of Hydrocortisone.* Exper. *14*, fasc. 10, 371 (1958).

There has been an error in the first column of page 372 in line 9 from below. This line should read: ... treated with hydrocortisone in concentration of 125 micrograms/ml.



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